RECENT STUDIES IN YEAST

CFTRI-MYSORE
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Recent studies i.

RECENT STUDIES IN YEAST
AND THEIR
SIGNIFICANCE IN INDUSTRY

Comprising
papers (with discussion) read at a Symposium
organized by the Dublin and District Section,
The Microbiology Group and the Food Group,
held in Dublin
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FIRST SESSION

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Chairman: MR. H. J. BUNKER

PHYSICAL PROPERTIES OF BREWING YEAST

by

A. A. EDDY

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As is well known, the more familiar range of brewing yeasts belonging to the species *S. cerevisiae* and *S. carlsbergensis* includes strains differing widely in brewing properties, particularly as regards the detailed physical behaviour of the yeast cells when suspended in nutrient media such as wort. The properties in question involve (*a*) the ability of the cells to aggregate, or as it is often termed, to flocculate; (*b*) the ease with which they are entrained by bubbles of carbon dioxide and carried to the surface of the fermenting wort, behaviour on which is based the distinction between 'top' and 'bottom' yeasts; and (*c*) their ability to precipitate isinglass, a reaction used particularly in British breweries to remove unwanted yeast and proteinaceous matter from turbid beer. It will readily be appreciated that the importance of these properties arises from the fact that they control the extent to which the yeast is maintained in contact with the wort at various stages during the fermentation and thus, indirectly, the eventual composition of the fermented wort or beer. It must be admitted, however, that we are relatively ignorant of the precise factors determining properties (*a*) and (*b*) which evidently depend on subtle aspects of the structure of the surface of the yeast cell.

A whole body of new evidence[1] has recently come to light in these laboratories on the basis of which it must be supposed that the flocculation of yeasts is a reversible process involving interaction between specific structures located in the cell surface, for the cells of flocculent yeasts are not simply 'sticky' in the sense that they adhere indiscriminately to all solid surfaces. They show, rather, a marked preference for attaching themselves to other yeast cells and here again further restrictions operate as only certain kinds of yeast are found to interact. The simplest case is where the cells of one yeast flocculate each other but fail to react with foreign cells. Various self-flocculent yeasts of this kind may be distinguished by the changes in flocculation which occur when the acidity is changed, when specific sugars are added and, finally, by the effect of ethyl alcohol on the intensity of flocculation. A somewhat broader specificity is shown in the phenomenon of mutual flocculation in which the cells of one yeast besides attracting each other also attract those

of certain other yeasts, which themselves may or may not be self-flocculent. Indeed, cases are known where neither member of a pair of mutually flocculating yeasts is self-flocculent to an appreciable degree. Such considerations have proved to be important in understanding the complex behaviour of the mixtures of yeasts which are often used in British breweries. For instance, mutually flocculating yeasts may tend under the conditions of commercial practice to be selected as a group from among a given heterogeneous mixture of strains in the course of successive fermentations.

Turning now to property (*b*) of yeast, namely that of collecting at the surface of the fermentation vessel, this has been shown to depend essentially on the ability of the cells to collect at the interface between the liquid phase and the escaping bubbles of carbon dioxide. The latter, incidentally, may conveniently be replaced by air in laboratory experiments. The tendency of top yeasts as opposed to bottom yeasts to collect in the interface may in fact be demonstrated by quite simple means, for instance by inspecting the surface of a drop of an appropriately buffered suspension of the cells.[2] It seems that even non-flocculent yeasts may form the yeast 'head' typical for top yeasts although, as might be expected, attractive forces between the cells may help to stabilize a film of yeast at the surface and, incidentally, draw into the surface other strains of indifferent head-forming ability.

In seeking a deeper explanation of these phenomena one is naturally led to explore the finer details of the structure of the cell surface, for it is clear in a general way that the specific forces which seem to govern the mechanical behaviour of suspensions of yeast cells originate in a specific structure of an unknown nature. One method of approach to this problem has consisted in studying the migration of various strains of yeast on being placed in an electrical field. An interesting preliminary conclusion drawn from these studies,[2] and from further studies using chemical techniques, is that phosphate groups play an important part in the structure of the outermost surface of the cells of many brewing yeasts. Furthermore, in many but not all cases they are found to be responsible for the major part of the negative charge located at the surface. No evidence has been found, however, that the phosphate groups as such play a part in flocculation or in the development of a yeast 'head', but their presence at the surface seems to be essential if rapid fining is to occur [the property mentioned above under (*c*)]. As first demonstrated by Wiles[3] and now confirmed in detail by work in these laboratories,[4] fining results from interaction between positively-charged particles of the collagen isinglass and negatively-charged

yeast cells. It is worthy of note that an analogous process of precipitation of positively-charged by negatively-charged yeast cells can be demonstrated but that the conditions under which the reaction takes place are not those which favour flocculation as it commonly occurs.[2] Indeed the latter, as already mentioned, would seem to be the expression of more specific forces of attraction, but until more is known about the nature of these forces it seems unlikely that an explanation will be forthcoming of the variations in flocculation which are commonly encountered in the brewery.

References

[1] Eddy, A. A., *J. Inst. Brew.*, 1955, **61**, 307, and unpublished observations
[2] Eddy, A. A., & Rudin, A. D., in preparation
[3] Wiles, A. E., *Proc. European Brew. Convention* (Brighton) 1951, p. 84
[4] Rudin, A. D., *J. Inst. Brew.*, 1956, **62**, 414

Discussion

Mr. J. H. St. Johnston: Can mutual flocculation of two types of yeast occur between a flocculent and a non-flocculent yeast and would that perhaps account for the regularity of proportion of flocculent and non-flocculent yeasts separated by the Burton Union system of fermentation?

Dr. Eddy: Yes it can and, as you suggest, may account for the behaviour you mention.

Mr. R. B. Gilliland: What effect would a small quantity of beer have on the differential character of the lysine medium?* Would it be possible to plate out a small quantity of sediment from a bottle of beer?

Have you done any quantitative recovery experiments to find if a small percentage of wild yeasts in a mixture could be accurately estimated?

Dr. Eddy: I think one would be advised to remove even traces of beer by washing in the centrifuge. Recovery experiments indicate that the wild yeasts are accurately estimated in the presence of excess brewing yeast.

Mr. A. E. Wiles: I note that the lysine medium, whilst effective in many cases, failed to detect some troublesome wild yeasts, e.g., strains of *S. carlsbergensis*. Is there any correlation between the mutually flocculating A and B types and their electrophoretic behaviour?

* Used for detection of contamination of brewing yeast strains with yeasts of foreign genera.

Dr. Eddy: Yes, the first comment is perfectly true. No correlation has so far been found between the A and B types and their respective electrophoretic behaviour.

Dr. J. I. Webb: What is the effect of lysine on the growth of Brettanomyces?

Dr. Eddy: All the strains tested have been found to grow.

Mr. M. H. Proctor: In the two groups of yeasts, (*a*) those showing a strong negative charge and (*b*) those with no charge or positively charged, is there any obvious difference in type or quantity of nucleic acid in the cell or specifically in the cell wall?

Dr. Eddy: Our studies of the composition of the cell wall have not proceeded sufficiently for me to be able to answer this question, but speaking from the experience of other workers I would say that there is little nucleic acid in the wall itself.

Dr. F. Winder: If the tendency of the top yeast to rise is to be attributed to a tendency to collect in the air/water interface, this presumably is due to a hydrophobic surface on the cell. Is this supported by a tendency to collect in an oil/water interface? The fact that the collection on the surface increases as fermentation progresses is in keeping with the fact that the lipid content of micro-organisms tends to increase as the culture ages.

Dr. Eddy: I have attempted experiments of this kind, using olive oil droplets in water suspension, but have so far failed to find evidence of accumulation at the interface. As regards your second point: I do not think that one can generalize on the matter of lipid content having regard to the variety of substances covered by the term 'lipid.'

RESPIRATION AND THE GROWTH OF YEAST

By PIOTR P. SLONIMSKI

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Introduction

In spite of numerous investigations concerning respiration and growth of *Saccharomyces cerevisiae*, certain fundamental relations between these two processes have been neglected almost completely in the past. The purpose of this paper is neither to give a general review of the question nor to discuss what is well known about respiration, growth and their mechanisms, but to bring out some of the salient discrepancies between the currently accepted picture and the experimental facts.

1. Measurement of respiration during growth

The manometric estimation of oxygen consumption using either Warburg or Barcroft respirometers is one of the techniques most widely used at the present time. In the early days of manometric techniques, it was found that the amount and the surface area of alkali employed for absorbing CO_2 in the Warburg procedure have to be quite large in order to permit a rapid absorption of the CO_2 evolved during vigorous fermentation,[1] and some thirty years ago Dixon[2] introduced the use of filter papers placed in the alkali centre well for this purpose.

Apparently it has not been realized, however, that this method presents a systematic error, irrespective of the concentration and surface of the alkali used. This error is due to the kinetic properties of the system and cannot be avoided. The extent of the error can be estimated precisely, however, and adequate corrections can, if necessary, be made.

In a Warburg vessel the rate of absorption of CO_2 by alkali follows a first-order equation

$$d[CO_2]/dt = K_1[CO_2]$$

The constant K_1 depends on the volume of the vessel, the alkali concentration and surface area, temperature, rate of shaking etc., but it should not change in the course of the experiment. Assume for a moment that the CO_2 output in a yeast fermentation is linear in time, i.e. $+d[CO_2]/dt = K_2$. An equilibrium will be established and the manometer will show no deflection if

$$+ d[CO_2]/dt = - d[CO_2]/dt.$$
$$K_1[CO_2] = K_2, \text{ or } [CO_2] = K_2/K_1.$$

This means that there will always remain a stationary level of CO_2 in the vessel even in the presence of alkali and that this stationary level may vary considerably during the experiment. When the rate of CO_2 evolution (K_2) changes, the stationary CO_2 level changes and the manometer shows a deflection which, in the case of oxygen-consumption measurements, will be attributed to changes in the respiratory rate. Variation in rate of CO_2 evolution unavoidably affects the determination of respiration.

For those not working with yeast, the error is an extremely small one, because the rates of CO_2 output and O_2 uptake are of the same order of magnitude, but it may be considerable for those interested in yeast fermentation problems.

Under usual conditions (vessel of 20 ml. total capacity, 2 ml. of buffer 4·5, 110 oscillations per minute, temperature 28°C, using 0·2 ml. of 20% KOH plus filter paper), the K_1 values are of the order of 0·3 – 0·4 min.$^{-1}$. If we place in the vessel 1 mg. dry weight of yeast having initially a Qco_2 of 360, then

$$[CO_2] = \frac{360}{60 \times 0{\cdot}3} = 20\mu l.$$

If, in the course of experiment the rate of fermentation increases by one half, an underestimate of 10 μl. of O_2 uptake will be made. If a brewer's yeast with a Qo_2 of, say, 20, is used in the experiment, the error will be 50%. It is easy to calculate that the error is important only if the rate of CO_2 evolution is several times higher than the rate of O_2 uptake and if there is a change in the CO_2 rate. And this is precisely what happens when yeast is growing. There are several possible ways to obviate the difficulty, such as to estimate the error and correct for it, to try to increase the constant K_1 by putting e.g., alkali into side-arms, or to measure oxygen uptake by a quite different method, e.g., an amperometric one.[3]

2. Influence of respiration on growth

It is a truism that a respiring yeast grows better than a purely fermenting one, by which it is generally implied that both parameters of growth,[4] the rate (measured during the exponential phase) and the yield (measured by the ratio of cellular matter synthesized to organic matter used up), are higher in the presence of respiration than in its absence. The experiments can be performed in two ways: (*a*) by comparing aerobic and anaerobic growth of the same yeast (this is the usual way), (*b*) by comparing aerobic growth of a normal

strain and a respiratory-deficient mutant derived from it. Ephruss and his collaborators[5–9] have studied these 'petite' mutants which lack cytochrome oxidase and are therefore unable to respire.

If such an experiment be performed on freshly isolated mutants the expected result is obtained. Fig. 1A shows aerobic multiplication of normal and mutant yeast in a fully aerated medium in which glucose is the only limiting factor of growth. Both the rate and the yield are much lower for the mutant than for the parent strain. After a certain number of transfers, however, the rate of growth of the mutant increases and eventually becomes identical with that of the normal strain.[6,8,10,11] Fig. 1B shows the same two strains as before but after about 450 transfers corresponding to several thousands of cell generations; they differ no longer in the rate of growth but only in the yield. Now, in spite of this acceleration, the mutant has preserved all its fundamental metabolic and enzymic deficiencies. It still lacks cytochrome oxidase and the cytochrome-*c* reductases of succinate, of α-glycerophosphate and of DPNH. It is clear therefore that a new, more efficient type of fermentative metabolism has been developed which, without consuming oxygen, suffices to assure the maximal growth rate. We know neither the

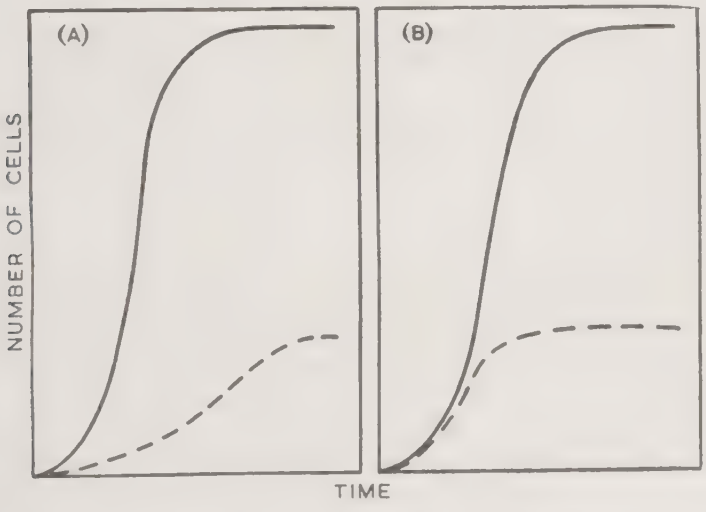

FIG. 1. *Rate of multiplication of normal and mutant yeasts.*

———Normal haploid strain 59R.

– – – – respiratory-deficient mutant derived from it 59RA.

Aerated medium containing glucose as the sole growth limiting factor.

A: freshly isolated mutant, B: the same, after approx. 450 transfers in pure culture. Schematic, after references 6 and 10.

biochemical basis of this phenomenon nor the mechanism of training which *a priori* may be due to selection of fitter respiratory-deficient mutants or to some physiological adaptation. It should be pointed out that, in natural conditions, this phenomenon could very rarely appear, if at all, because the 'petite' mutants would be wiped out by selection before the training could occur. It would appear that these fast-growing, permanently non-respiring 'petite' strains may be of great interest for some practical problems discussed at this Symposium.

Quite an analogous situation is found by studying anaerobic growth of normal yeast. Before discussing it, however, some remarks concerning anaerobic growth seem necessary. It should have been apparent from a careful examination of Pasteur's data[12] that the low yields of anaerobic growth could not be explained solely by the low energy-efficiency of sugar fermentation. Pasteur found in certain experiments that the yield of yeast in anaerobiosis was 50 to 100 times smaller than in the presence of oxygen. A purely energetic difference could have explained a difference of a factor of 15 at most, but not a factor of 100 (cf. references 6 and 13). This discrepancy is due to the fact that anaerobiosis increases the requirements of vitamins and growth factors. For instance,[6] a strain that grows aerobically *ad infinitum* in a synthetic medium containing only biotin and β-alanine does not grow for more than a few cellular generations in the same medium in anaerobiosis. Addition of several water-soluble vitamins stimulates the growth for a considerable number of cellular generations but does not assure an unlimited proliferation, which requires the addition of yeast extract to the medium.

Thanks to the work of Stier and his co-workers[14,15] we know now that *S. cerevisiae* has a mandatory requirement for sterols and other lipid-soluble growth factors for strictly anaerobic multiplication. Molecular oxygen seems necessary for their biosynthesis even under non-proliferating conditions.[16] From experiments done with cytochrome-oxidase-deficient mutants, it is concluded that in this respect oxygen plays a rôle not related to the rate of its consumption. The latter, although diminished by some 97–98%, is amply sufficient to permit unlimited proliferation of the mutant in synthetic media not supplemented with sterols. We have to distinguish therefore between two ways by which oxygen affects yeast multiplication. In the first, the major one, it acts as a terminal acceptor for electrons and completely changes the pattern of energy production in the cell. In the second, its action is nearer that of a trace element or a growth factor.

Harris[11] in this laboratory has confirmed Stier's results that normal yeast can grow *ad infinitum* under anaerobic conditions when supplemented with ergosterol and Tween 80. The experiments were done in an apparatus (cf. reference 15) which permitted all manipulations to be performed in an atmosphere of nitrogen containing less than 0·00005 parts of oxygen. In the course of successive transfers amounting to several hundreds of cell generations, a considerable increase of the growth rate was observed.[11,17] The final anaerobic value was quite similar to the maximal one in air. Such a rapidly growing anaerobic yeast is, however, perfectly capable of respiratory adaptation, i.e. it is able to synthesize all the lacking respiratory enzymes when exposed to oxygen. It contains only a very small proportion (approx. 1%) of 'petite' mutants.

The question arises whether training of the mutant and training of the normal yeast are similar. In the author's laboratories, normal baker's yeast has been trained to grow anaerobically almost as fast as it grows aerobically, and also respiratory-deficient mutants to grow aerobically as fast as does the normal strain. These two cases appear to be parallel, but we know now that they are different. Normal yeast (anaerobically fast-growing) gives rise occasionally to 'petite' mutants, which have the slow aerobic growth rate and not the fast one. Conversely, a 'petite' trained to grow fast aerobically will not grow as fast in the absence of air. In other words, at least two different mechanisms of supporting the maximal rate of cellular synthesis are compatible with the absence of respiration.

3. Influence of growth on respiration

If commercial baker's yeast is put in a buffer containing glucose, in the presence of air, it is found that the Pasteur effect operates and almost all the non-assimilated glucose is oxidized to CO_2 and water, ethanol is never formed in large quantities, the rate of respiration is very rapid and the rate of fermentation is slow. Since the appearance of Meyerhof's paper,[18] this classical picture is very well known to every student of biochemistry.

What happens if the same experiment is performed in a growth-sustaining medium? In other words, what is the influence of the general synthesis of cell materials on respiration and fermentation? Surprisingly enough, very few experiments have been done. It has been noticed by Meyerhof[18] and by Warburg[19] that yeast cultivated in the laboratory has in general a much lower respiration rate than commercial baker's yeast. Some ten years ago Swanson & Clifton[20] found that growing yeast first consumes oxygen slowly, then more rapidly. They concluded that young cells of baker's yeast, even cultivated aerobically, carry on their most active synthesis by

fermentative assimilation. This predominates until all the hexose has been used, oxidative assimilation then taking place from accumulated ethanol. Their data, although not always convincing in details (e.g. the manometric experiments were carried out under conditions where the systematic error of the method, discussed previously, must have been very large), are fundamentally correct. More recent carbon-balance studies by Lemoigne *et al.*[21] demonstrate that growing baker's yeast ferments glucose even if it is optimally aerated. During exponential growth, ethanol accumulates to the extent of more than 80% of hexose used and only in the last phase of growth which may show a diauxie phenomenon is ethanol respired. These findings seem paradoxical. Respiration of glucose is energetically more efficient than fermentation; oxidative assimilation as measured by intracellular carbohydrate synthesis is also much greater than the fermentative assimilation; the variety and versatility of carbon intermediates and building-blocks formed through the respiratory tricarboxylic acid and pentose cycles, is much greater than what can be produced by a relatively rigid Embden–Meyerhof–Parnas pathway. Last, but not least, the Pasteur effect which is so efficient in the absence of growth, should operate and prevent fermentation from gaining the upperhand. Yeast, however, grows wastefully most of the time, and at the end hastily dissimilates the alcoholic product of its own dissipation.

The reason for this lies in a regulatory mechanism of the yeast cell which has been called 'counter-Pasteur-effect'[13]. The Pasteur-effect describes a relationship between rate of fermentation and rate of respiration. It is a uni-directional relationship in which the first process depends on the second one and it operates at the level of enzyme *function*. The rate of fermentation does not influence, or influences relatively little, the rate of oxygen uptake (except for rendering the measurements more susceptible to the systematic error, *vide supra*). The counter-Pasteur-effect describes a relationship between the rate of synthesis of respiratory enzymes and the rate of fermentation, the former being in general inversely proportional to the latter. It operates at the level of enzyme *formation* and is therefore of great importance during growth and practically of no importance during an experiment of the type described at the beginning of the paragraph.

Respiratory enzymes in aerobic growth cycle

A study has been made of the fate of respiratory enzymes in the growth cycle.[22] Results of a typical experiment are given in Fig. 2. A baker's yeast with classical characteristics (spectrum showing strong bands of cytochrome *a*, *b* and *c*, high respiratory rate of

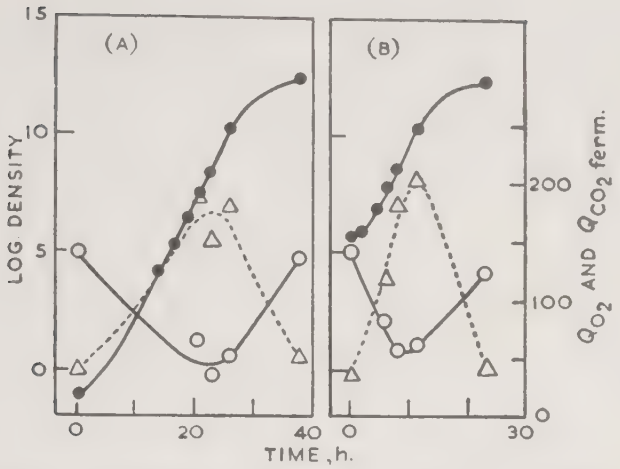

FIG. 2. *Evolution of metabolic parameters of normal yeast 59R growing in a synthetic, aerated medium containing 3% of glucose*

The parameters, $Q^{air}_{O_2}$ and $Q^{air}_{CO_2\,ferm.}$ are measured, under resting conditions, on washed samples and expressed per h. and per mg. dry weight of cells (from reference 22). Inoculum 100 times as great in B as in A.

● growth △ $Q_{CO_2\,ferm.}$ ○ Q_{O_2}

Q_{O_2} 140 and low aerobic fermentation of $Q^{air}_{CO_2\,ferm.}$ 38) is inoculated into a synthetic medium containing 3% of glucose. From time to time samples are taken out, cells are rapidly chilled and washed, their metabolic parameters are measured under standard resting conditions in the presence of glucose or ethanol, and finally the cytochrome spectrum is observed and certain enzymic activities assayed after breaking the yeast and extracting the enzymes. The two experiments shown in Fig. 2 differ only in that the size of inoculum in the first was 100 times smaller than in the other. In the first experiment the culture went through 14 cellular generations, while in the second one through only 7. The phenomenon was similar in both cases. From the commencement of growth the respiratory capacity fell, reached a minimum during the exponential phase and was finally restored to its original value during the phase of negative acceleration of growth. The $Q^{air}_{CO_2\,ferm.}$ varied in an almost exactly opposite manner. The Meyerhof quotient remains practically constant. The concentration of certain respiratory enzymes falls considerably during the exponential phase and explains the drop in Q_{O_2} values. Cytochrome *c* content is diminished by about 80%, as is that of cytochrome oxidase, but the concentrations of other constituents of the respiratory system do not vary in a parallel manner.

Glucose-6-phosphate dehydrogenase (TPN) and *iso*citric dehydrogenase (TPN) show no variation; while fumarase, alcohol dehydrogenase (DPN), succinic cytochrome-*c* reductase and lactic cytochrome-*c* reductase increase in amount at the end of the growth cycle.[23]

Experiments have been made to determine whether these changes are the result of (*a*) the destruction of enzymes already existing in the inoculum, (*b*) the complete arrest of new synthesis of enzymes with concomitant dilution in the growing mass of protoplasm or (*c*) the discrepancy between the rate of synthesis of respiratory enzymes and the rate of general protein synthesis.

The answer is given by Fig. 3A where the results are plotted in a different way (cf. reference 24): increment of the total amount of respiratory enzymes of the culture (ΔE) as a function of the increment of the total mass of the culture (ΔM). The curve is not horizontal, which means that the cells do synthesize respiratory enzymes during the exponential phase. Regression is linear within 2% limits of error, from which it is concluded that an equilibrium is established between the two rates of synthesis. The value of the slope gives the *minimal* value of Qo_2 that can be reached only when the enzymes already existing in the inoculum are sufficiently diluted by the growing mass of protoplasm. Further, this minimal value is maintained as long as the equilibrium is maintained. Approximately at

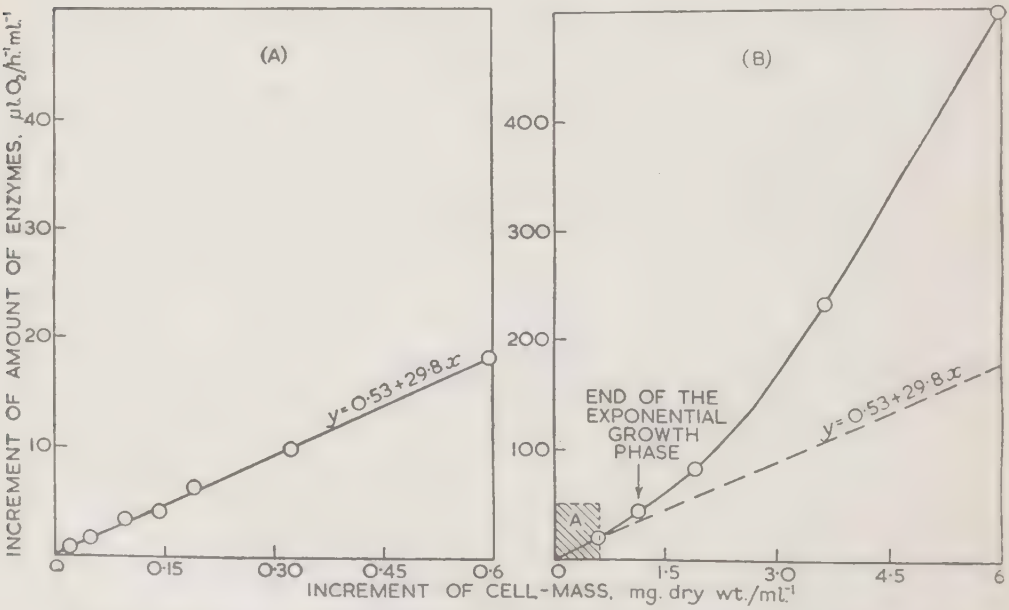

FIG. 3. *Evolution of the differential synthesis of respiratory enzymes during aerobic growth in a glucose-containing medium*

Co-ordinates are expressed per ml. of the culture. Modified, after reference 22

the end of the exponential phase or a little before it, the equilibrium is broken and a preferential rapid synthesis of respiratory enzymes takes place, resulting in the restoration of the original high Qo_2 (Fig. 3B).

It is known that some enzymes of terminal respiration are localized in mitochondria. It was of interest to compare the evolution of the metabolic and enzymic pattern with the one revealed by cytological observations, which was done in collaboration with Yotsuyanagi and others.[22] Fig. 4 shows the result of the experiment conducted for this purpose. The letters A, B, C, D. . . correspond to samples taken for microscopical studies. The yeast chondriome was

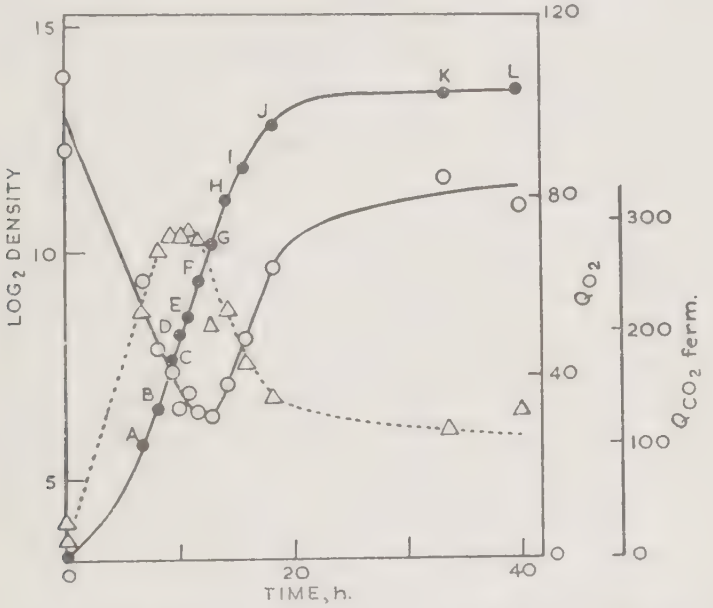

FIG. 4. *Evolution of metabolic parameters of a diploid yeast, strain Y.F., during aerobic growth in a yeast extract and glucose-containing medium* (e.g. Fig. 5; from reference 22)

● growth ○ Qo_2 △ Qco_2 ferm.

stained either vitally by Janus Green B or after fixation by the Altmann fuchsin. Evolution of the chondriome is quite remarkable and proceeds closely parallel to the physiological changes (Fig. 5).

Consideration has been given to the causes of the changes of enzymic constitution and of chondriome which may be due to inhibition of the rate of synthesis of respiratory enzymes by (*a*) the process of rapid cellular multiplication or (*b*) a constituent of the medium possibly accompanied by a rapid cellular multiplication.

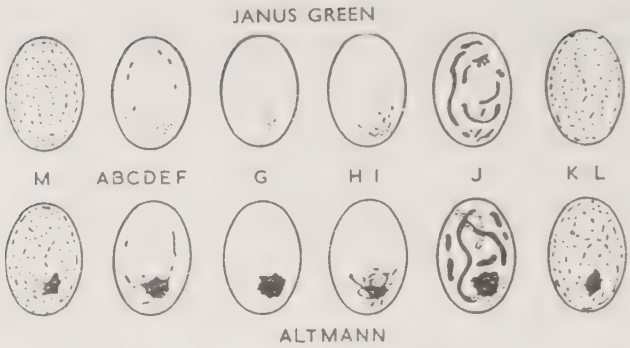

FIG. 5. *Evolution of the chondriome of yeast as revealed by two methods of staining*

The letters correspond to different samples as shown by Fig. 4 (from reference 22)

The first alternative implies a separation of the proliferation of the cells from the formation of the respiratory enzyme; the second does not.

Three types of experiments were done, two with growing cells, the third with non-proliferating yeast which was rapidly synthesizing cytochromes.

Three cultures containing respectively 1·0%, 0·25% and 0·08% glucose were grown aerobically from the same inoculum. The cultures were transferred to a fresh medium before the concentration of sugar had diminished by more than 20% of its original value. The cells grew exponentially with no interruption for about 35 cellular generations with practically identical rates for all the cultures ($0{\cdot}44\,h.^{-1}$, $0{\cdot}44\,h.^{-1}$, $0{\cdot}42\,h.^{-1}$). Nevertheless, their Qo_2 were not identical: 56, 73 and 98 respectively. The $Q\,^{air}_{co_2\,ferm.}$ varied in the opposite direction, viz., 186, 155 and 91 respectively, while the Q_M remain very similar. It seems therefore that the rate of synthesis of respiratory enzymes is inhibited by glucose rather than by the rate of proliferation.[22]

A yeast strain, grown on lactate as a sole source of carbon and energy for about 50 cell generations, was inoculated into glucose and into lactate media. In the first case it behaved exactly as previously described, the Qo_2 being minimal during the exponential phase. In the second case the effect was in the opposite direction. The synthesis of respiratory enzymes was *maximal* during rapid growth and the Qo_2 attained was exceptionally high, about 200. Glucose and not proliferation acted as inhibitor.[25]

Respiratory adaptation

An experiment on respiratory adaptation permits correlation of the inhibition with a metabolic parameter, the aerobic fermentation. It may be useful to recall some of the principal features of the adaptation phenomenon.[6,7,13,26,27] Growth in anaerobiosis suppresses the formation of several catalysts of the terminal respiration. In such an anaerobically grown yeast, molecular oxygen induces the formation of the whole chain of enzymes with consequent re-establishment of respiration. The synthesis of these enzymes may take place in resting conditions in glucose-containing buffer. Adaptation can be followed by several methods—measurements of catalytic activities of extracted enzymes, observation *in situ* of the characteristic absorption bands of cytochromes or measurement of the increment of the over-all respiratory rate. Fig. 6 shows that

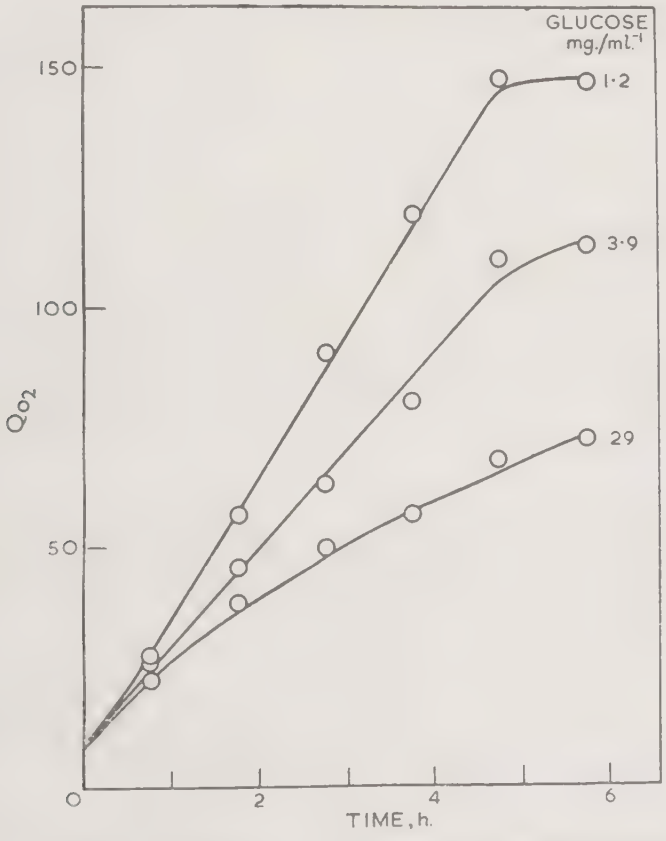

FIG. 6. *Inhibition of the respiratory adaptation by glucose*

Anaerobically grown yeast is rapidly washed and suspended in a buffer 4·5 pH containing indicated concentrations of glucose, and aerated. The rate of respiration is measured at successive intervals (Slonimski [17])

adaptation is slow when the glucose concentration is high. If the glucose concentration is too low, however, adaptation is diminished, which is quite understandable as being due to the lack of energy supply. By plotting the rate of adaptation as a function of initial glucose concentration, a relation is obtained that follows quite closely the one predicted by the plot of the rate of fermentation (acting as the inhibitor) *versus* glucose concentration (Fig. 7). The functioning of respiratory enzymes is almost unaffected by changes in the glucose concentration, the apparent dissociation constant for respiration being much smaller than that for fermentation.

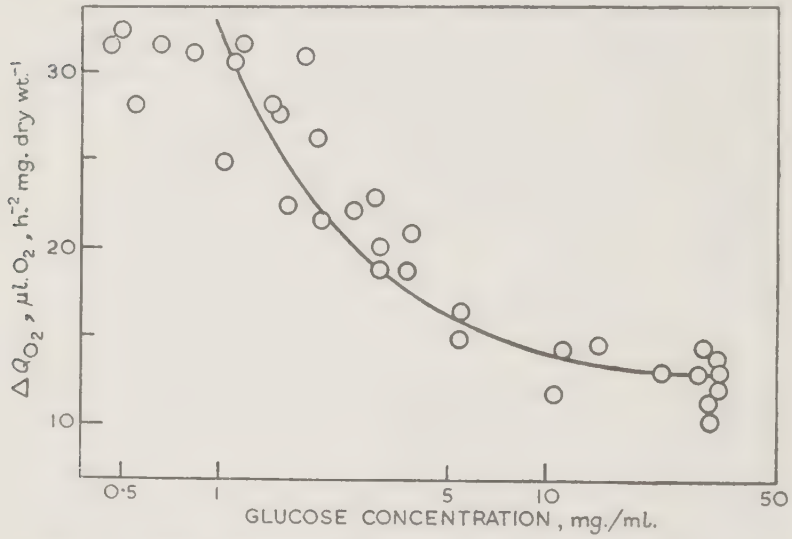

FIG. 7. *Rate of respiratory adaptation as a function of initial glucose concentration*

Constructed on the basis of experiments like the one given by Fig. 6 (from reference 13)

The following is suggested as the chain of events which follow inoculation of baker's yeast into a glucose-containing growth medium: high glucose concentration → high aerobic fermentation → slow differential rate of synthesis of respiratory enzymes → low respiration → low Pasteur effect → higher aerobic fermentation → slower rate of synthesis of respiratory enzymes → lower respiration → lower Pasteur effect etc. until a pseudo-equilibrium is reached which may not be distinguished experimentally from a true equilibrium. As the glucose concentration diminishes the sequence takes the opposite direction: low glucose concentration → low aerobic fermentation → high differential rate of synthesis of respiratory enzymes → high respiration → high Pasteur effect → lower aerobic

fermentation etc. At every moment of the growth cycle the metabolic properties of the cell are determined by the relative importance of the two regulatory and antagonistic mechanisms: the Pasteur effect and the counter Pasteur effect.

Conclusion

In the knowledge of the author, *Escherichia coli* is not produced commercially while *Saccharomyces cerevisiae* is. This is certainly very beneficial for human welfare, but it may become prejudicial to the understanding of yeast biology, since if one wants to know something about *E. coli* it has to be grown in the laboratory but, if a biochemist at the university wants a yeast he generally gets it from a brewery or a factory. Now the enzymic constitution, metabolic and cytological characteristics are so much dependent on the precise period of the cycle and conditions of growth that a number of apparently contradictory and conflicting observations may result. An obvious solution of this problem is to bring together as much as possible the people who grow yeast and those who grind yeast, so that this Symposium is an important step in this direction.

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Discussion

Prof. R. H. Hopkins: The observation that glucose in suitable concentration caused the yeast to synthesize less of the respiratory enzymes than otherwise was of interest. Analogous to it is the corresponding observation regarding the formation of certain hydrolytic enzymes, particularly the adaptive formation of, e.g., 'galactozymase and maltases; these are inhibited by glucose if present in more than very low concentrations.

Dr. Slonimski: Inhibition of the induced biosynthesis of enzymes by glucose is quite a general phenomenon and concerns enzymes belonging to very different classes. It is reasonable to admit, therefore, that the step sensitive to glucose is involved in some very essential part of the synthesis of a protein molecule.

Mr. G. Loggers: From Dr. Slonimski's experiments on the synthesis of respiratory enzymes, and from other experiments (e.g., by Davis in England) on hydrolytic enzymes, it follows that an appreciable synthesis of enzymes takes place, after most of the added glucose has disappeared, and actually only very little glucose is left. Will Dr. Slonimski comment on possible precursors for the enzyme formation in this case?

Dr. Slonimski: When there is no more glucose left there is still much ethanol that has accumulated during the exponential growth phase. Ethanol can serve as carbon and energy source. Nitrogen precursors may be looked for among the constituents of the intracellular pool of amino-acids, or perhaps ribonucleic acid could serve as a nitrogen reservoir for the synthesis of proteins.

Dr. A. A. Eddy: Did Dr. Slonimski find that the baker's yeast growing under anaerobic conditions actually required sterol; did the requirement persist after training?

Dr. Slonimski: (1) We have confirmed the findings of Stier and his collaborators concerning sterol requirements for anaerobic growth. There is probably a supplementary growth factor present in Tween 80 which is used to dissolve ergosterol. (2) Most probably, fast-growing, anaerobically trained yeast does still require sterols.

INOSITOL DEFICIENCY IN YEAST WITH PARTICULAR REFERENCE TO FAT PRODUCTION

By Dr. S. W. Challinor, N. W. R. Daniels and J. C. Hall

(*University of Birmingham*)

Introduction

During a study of the growth of a yeast (*Saccharomyces cerevisiae*) and a bacterium (a *Lactobacillus* species) in pure and in mixed culture in defined unaerated media containing sub-optimal amounts of inositol, interesting morphological and physiological changes were observed in the yeast growing in pure culture.

The deficient yeast cells formed large aggregates and were more granular than the 'complete cells' at 2 days and, after longer incubation, were very unhealthy in appearance and were easily ruptured; a large proportion of the cell contents consisted of material which gave typical staining reactions for fats when treated with Sudan III or with osmic acid.

Preliminary experiments

The deficient media used in the above experiments had been designed to support growth of both the yeast and the exacting lactobacillus and is complex. It contained a full complement of growth factors, eighteen amino-acids, purine and pyrimidine bases (adenine, guanine and uracil), 6% of glucose, salts, including a rather high concentration of sodium acetate (0·5%), and a sub-optimal concentration of inositol (approx. one-twentieth of that present in the complete medium). The amino-acids were later replaced by diammonium phosphate (0·237%). A representative experiment in the modified media will now be described.

The deficient medium contained only 0·6 μg. of inositol per ml., i.e. 6% of that present (10 μg./ml.) in the complete medium. The yeast was grown at 30° in culture bottles (Glaxo pattern) of 2·5-l. capacity, each containing 300 ml. of medium (depth of medium = 1·5 cm. approx.). After 5 days' incubation, the cells were harvested, ground in a Griffiths tube and dried. The fatty substances were then extracted at room temperature with light petroleum (five extractions). Results are shown in Table I.

The yeast grew moderately well in the inositol-deficient medium; the yield of fatty material from the 'deficient' yeast was approx. 8 times as great as that from the 'complete' yeast. The inositol-

Table I

Comparison of complete and inositol-deficient media

Medium	Yield of yeast, mg.	Yield of fatty material, mg.	Fat in the the yeast, %
Complete	489	7·9	1·6
Inositol-deficient	274	67	24·5

deficient yeast contains 24·5% of fatty material, i.e. approx. fifteen times as much fat as the yeast from the 'complete' medium. These preliminary results on small quantities of yeast may be subject to appreciable error and later experiments (in somewhat different media), indicate that the ratio may be of the order of 8—10:1. It is clear, however, that, under conditions of inositol deficiency the fat content of the yeast is greatly increased.

The fat content of the inositol-deficient yeast was not affected when the nitrogen-carbon ratio of the growth medium was decreased by lowering the concentration of ammonium phosphate in the medium by 50%.

Apart from their high fat content the inositol-deficient cells are of interest in that they are easily ruptured and extraction of the fat is therefore readily accomplished. Thus, in the above experiments extraction of the ground cells with light petroleum was effected at room temperature. Unground cells, i.e. cells which had no pretreatment, extracted under these mild conditions liberated 91% of their fat. The ease with which fat may be extracted from inositol-deficient yeast would be a factor of importance if this method of fat production were to be attempted on an industrial scale.

Simplification of the medium

The media used in the above experiments, although simpler than that in which the original observations were made, is still complex in nature. It contains a number of substances which are not essential for the growth of the yeast, namely the purine and pyrimidine bases, sodium acetate, and the growth factors nicotinic acid, pteroylglutamic acid and thiamine. Omission of all these substances from the inositol-deficient medium resulted in increased growth of the yeast, but decreased its fat content. Evaluation of the precise function (if any) of these substances in promoting fat formation in yeast is a task of some magnitude and has not yet been completed. For example, sodium acetate functions as a buffer in the medium

but it may also be concerned in fat formation. Yeast with high fat content was obtained, however, when the sodium acetate was replaced by potassium dihydrogen phosphate (1%). The other substances mentioned, with the exception of thiamine, could be omitted without apparent effect on the fat content of the yeast. The composition of the simplified *complete* medium is given in Table II.

Table II

Simplified complete medium

Carbohydrate	*per ml.*	*Salts*	*per ml.*
Glucose or sucrose	0·06 g.	$MgSO_4,7H_2O$	0·001 g.
		KH_2PO_4	0·012 g.
Nitrogen source		$CaCl_2,6H_2O$	0·00025 g.
$(NH_4)_2HPO_4$	0·00237 g.	$MnSO_4,4H_2O$	0·00001 g.
		KI	0·0000001 g.
		*Trace element solution**	0·001 ml.

Growth factors	*per ml.*
D-biotin	2·2 mμg.
Calcium D-pantothenate	2·4 μg.
Thiamine hydrochloride	2·0 μg.
Pyridoxine hydrochloride	2·4 μg.
Inositol	10 μg.

*Emery, McLeod, & Robinson, *Biochem. J.*, 1946, **40**, 426
The pH is adjusted to 5·5 with KOH

Deficient media contained reduced amounts of inositol or none at all. In the experiments to be described, the yeast inocula were grown in wort for 24 h. at 30°, harvested by centrifuging and washed twice with the inositol-deficient medium. A measured volume of a suspension of the washed yeast was added to give 3·3 μg. of yeast (calculated as dry matter) per ml. of medium. The cultures were incubated at 30° for five days except when stated otherwise.

Effect of variation of inositol concentration on yeast growth and on fat production

The inositol concentration was varied widely and media were prepared containing 0%, 6%, 10%, 20%, 40% and 70% of that in the complete medium; i.e. the concentration of inositol in the complete medium was regarded as 100%. Harvesting of the yeast was carried out when glucose utilization was complete. Results obtained in some of these media are summarized in Table III.

The general findings are as follows: Increase in inositol concentration gave increased yields of yeast but decreased its fat content and also the total yield of fat per culture. Yield of fat and the fat content of

Table III

Effect of variation in inositol concentration

Inositol concn., %	Yield of yeast per culture, g.	Yeast produced as % of glucose utilized	Fat in yeast, %	Yield of fat per culture, mg.
0	0·349	1·9	24·4	85·3
6	0·39	2·2	21·0	81·4
10	0·42	2·3	15·9	67·4
100	0·64	3·6	3·9	24·8

the yeast were maximal in the media containing no added inositol and were near maximal when the inositol concentration was 6% of that in the complete medium.

Growth of yeast and fat production in media containing no added inositol

The results just given directed attention to media containing *no* added inositol and further experiments in such media were therefore carried out. In the work so far described, the yeast was cultured in shallow layers of depth approx. 1·5 cm. It is obvious that the ratio of surface area to volume may affect both the growth of the yeast and its fatty content. The effect of varying the volume of the medium in the 2·5-l. bottles was therefore examined and the results are shown in Table IV.

Table IV

Growth of yeast and fat production in inositol-deficient media (no added inositol)

Volume of medium, ml.	Yield of yeast per culture, g.	Yield of fat per culture, mg.	Fat in yeast, %	Fat as % of glucose utilized	Amount of glucose utilized, g.	Final pH
300	0·39	65	18·2	0·4	18	3·4
600	0·89	69	8·5	0·2	36	3·4
1000	1·4	93	6·8	0·15	60	3·3

Yield of yeast increased with the volume of the culture, but the fat content of the yeast varied markedly at the different volumes, and decreased as the volume increased. The glucose was completely utilized in all these cultures, but the percentage conversion of glucose to fat was very low.

Effect of thiamine on yeast growth and on fat synthesis

Thiamine is not required for yeast growth and is, in fact, appreciably inhibitory under conditions of inositol deficiency. Under these

latter conditions, however, thiamine appears to play an important part in fat synthesis by the yeast.

Observations on the cell walls of the 'deficient' and the 'complete' yeast

The ease with which the inositol-deficient cells were ruptured, as compared with the cells of the 'complete' yeast, focused attention upon the cell walls. The cells were harvested by centrifuging, washed with distilled water and broken down in the Mickle tissue disintegrator (two treatments of 20 and 10 min. duration). The cell walls were washed with water until free from cell debris and from the few remaining whole cells.

Electron photomicrographs of the walls of each type of cell were taken (see Figs. 1 and 2). The photograph of the wall of the 'complete' cell is materially different from that of the 'deficient' cell. Thus, the wall of the complete cell appears to be more robust and its fracture is 'cleaner' and less extensive than that in the wall of the deficient cell. A 'network' is clearly visible on the wall of the 'complete' cell, but no such 'network' was seen on the wall of the 'deficient' cell.

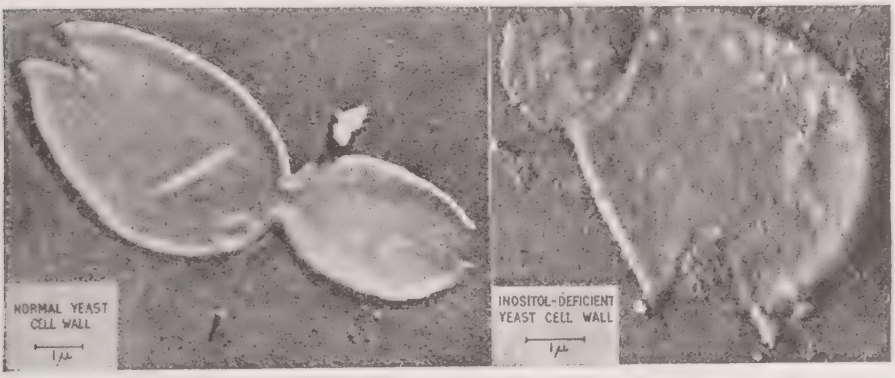

FIG. 1. *Normal yeast cell wall*

FIG. 2. *Inositol-deficient yeast cell wall*

Hydrolysates of the cell walls were prepared (hydrolysis with N-HCl for 48 h. in a sealed tube at 120°) and preliminary observations were made as summarized in Table V.

The walls of each type of cell contained appreciable amounts of protein, the content in the 'deficient' cell walls being appreciably lower than those in the 'complete' yeast; the protein of the latter appeared to contain the full range of amino-acids. The 'deficient' cell walls had the lower phosphorus content. A chromatogram of the

Table V

Observations on the cell walls of (1) *the 'deficient' and* (2) *the 'complete' yeasts*

Yeast	Protein, %	Phosphorus, %	Inositol, μg./mg.	Chromatography	
				Amino-acids	Sugars
'Complete'	17·0	0·22	<0·25	A full range	Two unidentified; only a trace of glucose
'Deficient'	13·2	0·18	—	—	—

hydrolysate of the wall of the 'complete' cell showed only a trace of glucose. The 'complete' cell wall contained an insignificant amount of inositol as judged by microbiological assay (using *Schizosaccharomyees pombe*).

Conclusion

As was stated at the outset, interest in the morphological and physiological changes which occur in yeast when grown in inositol-deficient media was stimulated during an investigation of the growth of the yeast and a bacterium in mixed culture. This latter work is difficult and time-consuming and the time available for the study of the inositol-deficient yeast in pure culture has been limited, but the results have proved of great interest.

Little is known concerning the nature of the fat content of the inositol-deficient yeast. The fat extracted is a pale yellow viscous oil with a saponification value of approx. 130 and unsaponifiable matter of the order of 30% (the latter estimation was carried out on the residue from the sap. val. determination and is therefore very approximate).

Yeast with high fat content was obtained when sucrose was substituted for glucose in the inositol-deficient media. Preliminary experiments in which the yeast was grown in *stirred aerated* culture in inositol-deficient media (containing glucose or sucrose) were carried out and it was found that the fat content of the inositol-deficient yeast was much higher than that of the corresponding complete yeasts.

The high fat content of *S. cerevisiae* when grown in inositol-deficient media has been clearly established. Is this a general phenomenon applicable to other inositol-requiring yeasts? Smith[1] has studied the role of inositol in the nutrition of *S. carlsbergensis* but he did not comment on fat formation. Two other inositol-

requiring yeasts (strains of *S. carlsbergensis* and *Kloeckera brevis*) have also been examined in inositol-deficient media. The fat contents of the yeast so obtained were much higher than those of the corresponding 'complete' yeasts.

(Some of the earlier findings recorded in this paper have been reported[2] briefly elsewhere.)

Acknowledgments

The authors are indebted to Professor Sayers of the Electron Physics Dept., for permission to use the electron microscope and to Mr. C. C. Newton for his help in the preparation of the electron micrographs. They also wish to thank the Department of Scientific and Industrial Research for a grant to one of them (N.W.R.D.).

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Discussion

Dr. P. Slonimski: I would like to mention the work done by Mrs. H. de Robichon-Szulmajster from our laboratory, concerning interactions of pyrimidines with inositol. She has found that among strains of *Saccharomyces cerevisiae* requiring *meso*-inositol there was a special class that required it only if the pyrimidine supplied was uracil, but did not require inositol if the pyrimidine was cytosine. We may have here the first precise results as to the mechanism of action of inositol. Did Dr. Challinor study the action of pyrimidines in his inositol requiring strains?

Dr. Challinor: No.

Dr. F. Winder: It is known that a glycerophospho-protein complex is a component of the cell wall of some bacteria. I wonder whether an inositol phosphoprotein complex could play the same part in yeast cell walls?

Dr. Challinor: It is possible.

Mr. H. J. Bunker: This connexion between inositol deficiency and fat formation should be of interest to those working with the so-called fat-producing yeasts, such as those investigated by Kleinzeller, Starkey and Lundin and his collaborators. In these yeasts, fat formation occurs when nitrogen starvation arises, but there is no pathological development in the cell wall in those yeasts. I do not know whether these fat-producing yeasts are inositol-deficient.

Dr. L. A. Allen: Following on Mr. Bunker's remarks I would say that the regular fat-forming yeasts did *not* require inositol. I should like to ask Dr. Challinor whether, in the inositol-deficient medium *Saccharomyces cerevisiae* assimilated ammonium to the same extent as it did in the complete medium?

Dr. Challinor: When nitrogen was reduced by 50% there was little apparent difference in fat production. We have not compared the nitrogen assimilation in the two media.

Dr. A. A. Eddy: I am rather surprised that Dr. Challinor had failed to discover glucose in the hydrolysate of the cell wall, a sugar which several other workers had previously detected. Is the failure to obtain glucose due to difference in methods of hydrolysis? A glucan constitutes 30% of the weight of the cell wall, but this is very resistant to hydrolyses.

Dr. Challinor: The hydrolyses were carried out in a sealed tube at 120°.

Dr. A. A. Eddy: Did the hydrolysates contain hydrolysed protein?

Dr. Challinor: I do not know.

Dr. H. Jørgensen: Could one of the unidentified sugars in the yeast wall possibly be mannose, originating from yeast gum in the wall?

In connexion with Dr. Eddy's remarks, I should like to draw attention to the fact that in yeast-dextran we possibly have β-1:3-linkages, whereas cellulose possesses β-1:4 linkages; β-1:3 linkages are probably very resistant to hydrolysis.

Mr. D. H. F. Clayson: Is the fat formed by yeast growing in inositol-deficient media different from that of cells grown in inositol-sufficient media in respect of its content of essential fatty acids? Attention is drawn to current work on the importance of essential fatty acids in connexion with atheromatous changes in animals.

Mr. R. P. Murphy: Has any work been done on rejuvenation of inositol-deficient yeast by addition of inositol?

What effect have inhibitors (e.g., azide, nitrophenol, etc.) on fat formation in deficient yeast?

Has a comparison of enzymic constituents of deficient and normal yeast been made?

Dr. Challinor: There is no information on these questions as yet.

SECOND SESSION

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Chairman: PROF. T. DILLON

AMYLASE SYSTEMS IN BREWERY YEASTS

By Prof. R. H. Hopkins

(*University of Birmingham*)

Introduction

Brewer's culture yeast does not ferment the saccharide products of amylolytic breakdown of starch other than the sugars, glucose, maltose and maltotriose.[1] The various culture yeasts in use may vary somewhat in this respect. No report is yet available that maltotetraose is fermentable by any of them and this saccharide may be regarded provisionally as unfermentable by primary yeasts. The higher saccharides and all which contain the α-1:6 linkage survive in beer.[2,3] Certain yeasts, however, have been found in and isolated from bottled beer which ferment beer dextrins causing a further fall in specific gravity in the storage vessel or bottle. The phenomenon has been described as 'super-attenuation'.[4] The yeasts *Sacc. diastaticus*, *Br. bruxellensis*[4,5] have marked powers in this respect.

During the life cycle of the yeast cell, glycogen successively appears and disappears, so that some metabolic mechanism for the utilization of glycogen, a food reserve, must be presumed. Earlier work can be interpreted in the light of the later discovery of phosphorylase and it was the opinion of Myrbäck that the fermentation of starch, glycogen, etc. by yeast juice and yeast extracts was initiated by the action of phosphorylase.

Long ago, experiments performed in Birmingham[6–8] resulted in the preparation from brewery yeasts of an amylolytically active extract. The activity was always low but it was definitely established as being due to β-amylase. This, however, has since been found not to originate from a secretion by the cells but to have been adsorbed by the cells from the wort or beer from which they were harvested.[9]

Amylases in brewing fermentation

Table I gives amylolytic activities (α and β) in an unboiled wort during fermentation on a technical scale.

It is seen that both α- and β-amylase disappear during fermentation. That they are to be found on the yeast cells is easily demonstrated by collecting the yeast from a brewery which adds 'yeast dressing' to the fermenting wort, and drying and extracting it. Many brewers make this addition in order to assist fermentation, clarification etc. and in particular to improve the vigour of the yeast itself, some of which is to be used for pitching a subsequent brew.

Table I

Amylolytic activity of (unboiled) malt wort during fermentation

Days of fermentation at 30°	Sp. gr. of wort (water 1000)	Amylase activity (Lintner degrees) α	β
0	1062	25	71
1	1022	1	5
3	1009	0·25	3·7
5	1004·5	0·1	3

Yeast dressings usually contain either wheat flour, which contains β-amylase, or malt flour which contains both α- and β-amylases. So far as the amylase is concerned it probably functions by splitting maltotetraose into maltose and, albeit slowly, maltotriose into maltose and glucose. In the brewery fermentation process, maltose and the other fermentable sugars disappear first, but the slow continuing fermentation of maltotriose in the concluding stages may interfere with the separation of the yeast. A more clear-cut end is often helpful in the brewery routine. The added amylase may help by virtue of its action on maltotriose, but it exercises a more definite influence during conditioning in that it brings about the liberation of more maltose, which is fermented to provide the additional pressure of carbon dioxide desired at this stage.

To return to this yeast, crops which have been harvested from worts containing either or both of the amylases will have some of the latter concentrated on the cell surface. If this yeast is then sown into sterile wort to which yeast dressings are not added, the adsorbed amylase may nevertheless exert a small influence in the manner indicated above. The presence of amylase on the cell surface may also be of significance if the yeast is used in the laboratory, e.g. for the determination of attenuation limit. For example, maltotetraose, which is not fermentable, could be split by the amylase and the resulting maltose fermented, giving a low final specific gravity in the laboratory test.

Isoamylase

Apart from amylases adsorbed on the cell surface of the yeast in use in certain breweries, no true amylase has been reported in culture yeasts. A starch-debranching enzyme, however, has been reported in brewer's yeast, both top and bottom types. It promotes hydrolysis of the α-1: 6-linkage in amylopectin and its β-limit-dextrin

irrespective apparently of the chain length of the branch removed. The enzyme was vaguely described by Maruo & Kobayashi[10] who called it 'isoamylase', further characterized by Hopkins[8] (who called it a debrancher) and later isolated and still further characterized by Manners & Khin Maung[11] who use the name 'isoamylase'. An analogue in the higher plants is the R-enzyme[12] in potatoes which detaches branches of two glucoside units or longer but not of single units. But 'isoamylase' combines these functions with those of α-1:6-glucosidase since it can also remove single glucose units.[8, 11] The enzyme functions at the reaction natural to the yeast cell, about pH 6, but not at pH 4·6. At about pH 6 it withstands a temperature of 50° for 15 minutes without inactivation (See Table II)

Fermentation of limit dextrin

The β-limit-dextrin left when β-amylase acts on amylopectin cannot be further split by the β-enzyme unless the dextrin is first attacked by either α-amylase or a debrancher. In the former case the iodine coloration is destroyed concurrently with the appearance of relatively little reducing power, but in the latter case the joint actions of debrancher and β-amylase give a linear relationship, and the destruction of the whole of the iodine coloration would be accompanied by the formation of 100% maltose. The latter, in the presence of yeast extracts, would in its turn be split by maltase to glucose. In Table II, hydrolysis of β-limit-dextrin by a preparation from brewer's (top) yeast containing β-amylase is shown, indicated by diminution of iodine coloration measured in a Spekker Absorptiometer. It is also shown that the activity was not impaired by 15-minutes' heating to 50°. Its action on amylopectin β-limit-dextrin is incomplete, about one-third of the branched molecule resisting hydrolysis. How far this is due to the closeness of some of the branches and how far to the presence of α-1:3-linkages[13] is not known.

Table II

Effect of heat on the debranching enzyme of brewer's yeast.

Brewer's yeast, air dried, extracted with water at 15° for 15 h. and the clear extract brought to 0·025M with $CaCl_2$. After heating portions to the temperatures stated for 15 min. and cooling, equal volumes were allowed to act on 0·5% β-limit-dextrin at 30° amd pH 6 (citrate buffer), excess of β-amylase being present.

	% *Fall in absorptiometer readings* (600 mμ)			
Temperature of preheating	15°	40°	50°	60°
After 18 hours	16	14	16	0
After 42 hours	33	33	37	0

By debranching, the enzyme can also collaborate with the phosphorylase of yeast to effect a considerable degree of break-down of the cell glycogen.

Transferase in yeast

A further enzyme, part of the system, is the branching enzyme, a transferase resembling the *Q*-enzyme of Haworth, Peat & Bourne.[14] This was reported briefly by Hopkins[8] and by Manners & Khin Maung[15] who separated it as a protein preparation by ethanol–citrate fractionation. This enzyme, in effect converts amylose (very long chains, unbranched) into amylopectin (highly branched). The branch point formed can be split by the debrancher.

Yeasts which ferment starch and dextrin

Of these, *Sacc. diastaticus* (Andrews & Gilliland) has received the most attention. Starch hydrolysis is effected by virtue of the amylase 'glucamylase'.[8] This enzyme splits glucose from the starch (amylopectin and amylose) and glycogen molecules,[16, 17] its action resembling that of β-amylase, except that the fission product is β-glucose instead of β-maltose. It has also been named 'amyloglucosidase'[17] and is secreted by *Aspergillus niger*, as well as by *Rhizopus delamar*.[16] It promotes the above action readily, the resulting glucose being fermented by the normal fermenting enzyme system. Glucamylase splits maltose relatively slowly. The evidence that the amylase present in the cells of *Sacc. diastaticus* is glucamylase and not, say, β-amylase accompanied by excess of a maltase, is provided by the extreme rapidity of its action on starch to produce glucose (which was shown on a paper chromatogram to be formed in 1 minute), accompanied by its relatively slow action on maltose. *Sacc. diastaticus*, acting as living yeast, can ferment starch[4, 8, 18] and it seems probable that the enzyme glucamylase is secreted at or near the cell surface—at sites to which colloidal starch etc. can gain access. There is also a debrancher present, apparently, but within the cell. This might be identical with the debrancher, 'isoamylase'. There is also a maltase present — apparently identical with the maltase 'M.1' of Winge & Roberts,[19] the maltase which can split sucrose as well as maltose by virtue of its specificity for the α-glucopyranoside group. The identity was established by Gilliland.[20]

The industrial significance of yeasts such as *Sacc. diastaticus* lies in (*a*) their potential use in the production of ethanol from starch materials, (*b*) their use in increasing the attenuation of beer, especially strong beers and beers made from grists which saccharify imperfectly in the mashing process, and (*c*) their nuisance value as pathogens in normal beer undergoing storage.

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Discussion

Mr. H. J. Bunker: Regarding the possible use of *S. diastaticus* in improving attenuation, it is my experience, and I believe that of others, that the flavour of the beer produced is very unpleasant.

Prof. Hopkins: That is a matter of taste.

Dr. K. G. McKenzie: (1) Have you ever detected any synthesis of oligosaccharides during fermentation, as well as the normal type of breakdown? (2) Has this type of work been done solely on yeast extracts or has live yeast been tested?

Prof. Hopkins: (1) Enzyme preparations have yielded traces of oligosaccharides (spots on paper), the origin of which may be synthetic, but in general no evidence of synthesis has been obtained.

(2) Living *S. diastaticus* ferments amylopectin slowly and incompletely (to the extent of 25% but it might well have proceeded further). Owing to the colloidal character of amylopectin it seems that the glucamylase must either diffuse out of the cells or be secreted at or near the surface at sites to which the amylopectin can pass.

Mr. D. H. F. Clayson: Does the amylolytic activity of *S. diastaticus*, together with the objections to its flavour mentioned by Mr. Bunker, suggest that amylases have a co-enzyme of a sulphydryl nature? It has been reported that the flavour of beer is to some extent due to trace amounts of mercaptans, which in larger amounts would, of course, be objectionable.

Prof. Hopkins: Many more α- and at least two β-amylases have been crystallized. No sign or indication of co-enzymes has been reported, although some amylases for their activity require the presence of certain ions, e.g., the calcium ion.

Prof. T. Dillon: Have you found the Z-enzyme of Peat *et al.* in yeast, the enzyme which hydrolyses β-glucosidic linkages of amylose in starch?

Prof. Hopkins: No sign of it has been found, but tests may not have been sufficiently searching. Amylose is difficult to handle—it retrogrades for instance—unless the enzyme is rapid in its action. My enzyme preparations and the living yeasts acted slowly, but acid hydrolytic fission products of amylose, staining with iodine, were effectively attacked, but whether the Z-enzyme was involved could not be stated.

YEAST CONTROL IN CIDER FERMENTATION

By F. W. Beech

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Introduction

Cider-making commences in the autumn when the fruit is milled and pressed, the expressed juice is allowed to ferment naturally and the dry cider is then stored until required for sale. The chemical composition of the juice varies according to the type of apples used and their source, while the juices contain a heterogeneous collection of yeasts, moulds and bacteria. Both of these factors affect the fermentation of the juice and the stability of the cider. Control methods are necessary for regulating the rate of fermentation, for stabilizing the ciders against further yeast growth during storage and sale, for preventing the development of undesirable bacteria, and, perhaps most important of all, for maintaining the quality of the final product.

The micro-flora and chemical composition of apple juices

An indication of the variety of yeasts and bacteria that have been isolated from fruits and fermenting juices is given in Tables I and II. Moulds isolated from apples and apple juices[1] include species of the *Mucorales*, *Aspergilli*, *Pencillia* and the *Fungi imperfecti*.

The variable chemical composition of the juice of different apple varieties, is in turn influenced for each variety by climate, the nature

Table I

Yeast isolated from apples, juices and ciders

Family Endomycetaceae	Family Cryptococcaceae
Endomyces mali[2]	*Cryptococcus albidus*[3]
Saccharomyces cerevisiae[3]	*C. neoformans*[3]
S. uvarum	*C. laurentii*[3]
S. microellipsoides[4]	*Candida pulcherrima*
S. heterogenicus[4]	*C. krusei*[5]
S. elegans	*C. malicola*[3]
S. florentinus[6]	*C. scottii*[3]
S. oviformis	*C. mesenterica*[3]
S. steineri[3]	*Torulopsis famata*[3]
S. bisporus[7]	*T. candida*[3]
Pichia membranaefaciens[3]	*Brettanomyces bruxellensis*
P. polymorpha[3]	*B. claussennii*
P. sylvestris	*Rhodotorula mucilaginosa*[3]
Debaryomyces kloeckeri[3]	*R. glutinis* var. *rubescens*[3]
Saccharomycodes ludwigii	*Kloeckera apiculata*[7]

Table II

Bacteria isolated from apple juices and ciders

Gram-positive	Gram-negative
Lactobacillus brevis[10]	*Acetobacter xylinum*[1]
L. pastorianus var. *quinicus*[10]	*A. oxydans*[1]
L. plantarum[1]	*Saccharomonas anaerobia* var. *pomaceae*[11]
L. delbrückii[1]	
L. leichmanni[1]	
Leuconostoc mesenteroides[10]	
Microbacterium sp.[10]	

of the soil and orchard practice. From a range of 100 single varieties tested at Long Ashton in 1953, extremes of specific gravity of the juices were 1·040 and 1·079; titratable acidity, 0·12% and 2·37% (as malic acid); tannin, 0·07% and 0·52%; soluble nitrogen, 2·2 mg. and 27 mg. per 100 ml. of juice; rate of fermentation, 0·1 and 8·2 (expressed as degrees of specific gravity lost per day at 25°). Within a single variety, Kingston Black,[12] the juice analyses over the years 1903-1937 varied as follows: specific gravity, 1·048 to 1·076; titratable acidity, 0·42% to 0·80%; tannin, 0·11% to 0·29% and rate of fermentation, 2·5 to 7·1. The ciders exhibit the same degree of variation as the juices from which they were derived, ciders balanced in flavour are obtained only by processing mixtures of apple varieties and by blending the final ciders.

Factors affecting rate of fermentation of apple juices

A knowledge of the factors controlling yeast growth and rates of fermentation in apple juices is necessary before any yeast control method can be developed. It has shown[13] that the soluble nitrogen content of fermenting juices first decreases and that this decrease is due to the uptake of nitrogen by the yeasts. The yeast crop develops to a maximum before active fermentation sets in and remains virtually unchanged until the end of fermentation, when it decreases slightly due to autolysis. These changes, calculated from changes in the soluble nitrogen contents of the juice during fermentation, are illustrated in Fig. 1.

Removal of most of the yeast crop causes a temporary cessation of fermentation, which despite the presence of adequate soluble nitrogen (for further yeast growth) may sometimes not be resumed without the addition of thiamine.[14]

The converse problem occurs with juices of very low soluble-nitrogen content: the fermentation is extremely slow or may even cease before all the sugar has disappeared. A steady rate of fermentation can be induced in such ciders by adding amounts of soluble

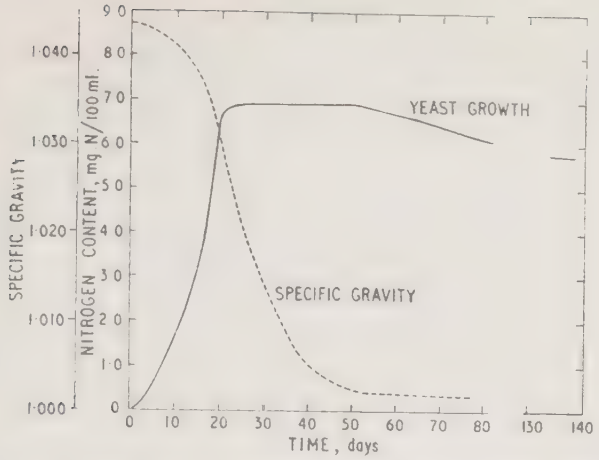

FIG. 1. *Yeast growth in a fermenting cider, as calculated from changes in the soluble nitrogen of the liquid*

nitrogen (in the form of ammonium salts) and thiamine just sufficient to bring about the required fall in gravity.[15]

Control methods based on reduction of nutrients

The rate of fermentation of cider can be controlled by reducing the nutrient status of the juice or partially fermented cider. Methods based on this principle are used for slowing down very rapid fermentations or for the preparation of naturally sweet ciders.

Keeving

Much of the cider produced in France is naturally sweet since artificial sweetening of dry ciders is not permitted. The process used is called 'défécation' or 'keeving' and basically it depends on reducing the soluble nitrogen content of the juice. The milled apple pulp is macerated, and is stored for several hours before pressing to increase the pectin and pectin-esterase content of the juice; the expressed juice is then held at 5° for keeving to take place. The soluble pectin is demethylated by the pectin-esterase in the juice[16] and is precipitated as insoluble calcium pectate if sufficient calcium ions are present; the jelly-like floccules coalesce and micro-organisms in suspension are entrained in the deposit. The temperature of the juice is then raised slightly, causing yeast fermentation to commence and raising the jelly to the surface to form a compact head. The clear juice, syphoned from between the head and lees, has a lower nitrogen content by virtue of the amount taken up for yeast growth in the first vessel.[17] Consequently the rate of fermentation of the keeved juice is slower than that of the untreated juice, as shown in Table III.

Table III

Effect of keeving on the soluble nitrogen content and rate of fermentation of apple juices.

Apple variety	*Treatment*	*Soluble nitrogen* mg. per 100 ml.	*Days to ferment to sp.gr.*1·025 *at* 12°
Sweet Alford	Untreated juice	4·0	48
	Keeved juice	1·6	180
Yarlington Mill	Untreated juice	4·8	41
	Keeved juice	2·5	85
Bulmer's Norman	Untreated juice	18·4	7
	Keeved juice	15·9	30

The treatment is less effective with juices of high soluble-nitrogen content.

Although other explanations have been advanced,[18] it is considered that the soluble nitrogen content of the juice is reduced first by yeast growth; the greater part of the yeast crop is then removed mechanically in the pectin jelly or is left behind in the lees. If this assumption is correct, then filtration of the fermenting juice should produce a similar effect, whereas inhibition of pectin coagulation, without yeast removal, should permit the same yeast growth as in the untreated juice. To test this theory the following juices were prepared:

(*a*) Untreated juice;

(*b*) Juice from macerated pulp (in duplicate);

(*c*) as (*b*) plus a pectin-destroying enzyme;

(*d*) as (*b*) plus a trace of calcium carbonate to assist keeving.

The juices were held at 2° and, when the jelly deposit had formed in (*d*), the temperature of the juices was raised to 10° until the jelly in (*d*) had risen to the surface. The juice was syphoned from (*d*) and at the same time one juice of treatment (*b*) was filtered on pulp. The effects of these treatments on the subsequent rates of fermentation of juices from two apple varieties are shown in Table IV; viable yeast counts for one juice (Sweet Alford) are given in Table V.

It will be seen that the yeasts in suspension first tended to decrease in all the juices at 2° due to sedimentation and then to increase again with the development of the main yeast crop (*Saccharomyces* spp.). The decrease was most marked in the juice undergoing keeving, but even here the numbers of yeasts increased as the jelly was lifted to the surface. The syphoned juice had a greater yeast population than the filtered and hence tended to

Table IV

Effects of keeving and filtration on rate of fermentation

	Juice treatment	*Days to reach sp. gr. 1·025 at 12°*	
		Red Foxwhelp	Sweet Alford
(*a*)	Untreated	128	68
(*b*)	Juice from macerated pulp	203	86
(*c*)	Juice from macerated pulp + pectin-destroying enzyme	130	60
(*d*)	Juice from macerated pulp after keeving	>260	263
(*b* filt.)	Juice from macerated pulp after filtration	>260	>260

Table V

Counts of viable yeasts in suspension (per 1 ml.)

Days after pressing-out juice	Untreated juice	Juice from macerated pulp	Juice from macerated pulp plus pectin-destroying enzyme	Juice from macerated pulp undergoing keeving	Observations on juice in keeving jar
	(*a*)	(*b*)	(*c*)	(*d*)	
0	1,345,000	635,000	675,000	1,080,000	
5	670,000	520,000	435,000	280,000	First signs of jelly floccules.
11	250,000	120,000	160,000	150,000	More jelly.
15	250,000	130,000	60,000	5000	Firm jelly deposit. Juice brilliant.
*21	6,750,000	965,000	2,850,000	935,000	Firm head on surface.
22	8,250,000	3,750,000 3500 after filtration	8,850,000	3,350,000	Syphoned off.

* Temperature of room raised to 10° on day 19

ferment slightly faster (Table IV). The juice in which pectin was destroyed by the enzyme, had finally the same number of yeasts in suspension and the same rate of fermentation as the untreated juice.

While the natural clarification reduces the number of yeasts, and therefore, the soluble nitrogen content of the juice, filtration after a short period of fermentation would be equally effective and less dependent on the pectin content of the fruit. In France it is found

that keeving does not always reduce the rate of fermentation sufficiently: it must be supplemented by low-temperature fermentation of the keeved juice and cold storage of the centrifuged sweet ciders.[19]

Centrifuging

In the past, the centrifuge was used mainly as a means of producing naturally sweet ciders, usually from juices of low or medium nitrogen content: it is now used more to regulate the rate of fermentation of high-nitrogen juices. The process depletes the soluble nitrogen content of the juice by an amount equal to that taken up by the yeast growth. The next crop is relatively small due to the lower nitrogen status of the centrifuged cider, so that the subsequent fermentation is slower. The second object of the method is the production of dry ciders with a low nitrogen content (1·5 mg. of N/100 ml. or less) that will not re-ferment when sweetened in trade casks.

There are two serious objections to this method. If the cider is centrifuged after only five degrees drop in specific gravity, the main fermenting yeasts are drastically reduced, whereas the smaller and less efficient *Kloeckera* spp. are hardly changed in numbers and compose the major part of the yeast flora of the centrifugate. If centrifuging is delayed until the later stages of fermentation, this objection no longer applies since most of these smaller yeasts have died out. The second objection to the method is that the ratio of yeasts to bacteria is seriously upset; this becomes more marked with the progressive increase in the numbers of bacteria in the later stages of fermentation. These changes in the micro-flora are illustrated in Table VI for ciders made from a juice with specific gravity 1·060 and pH 3·6.

The bacteria present in fermenting ciders are almost entirely anaerobic and their development is dependent on the pH of the cider.[20] At low pH (3·2) they grow very slowly indeed and, therefore, such ciders can be centrifuged with little danger of subsequent bacterial disorders. At higher pH (above 4·0), however, the anaerobic bacteria grow freely, and if the ciders have low nitrogen contents it is dangerous to centrifuge them while still sweet, since the lactobacilli will develop in the centrifugate before the yeasts and will produce lactic and acetic acids from sugars. This is one reason why naturally sweet ciders are now rarely produced in England. The low nitrogen content of the sweet cider will prevent yeast fermentation but, if the cider is of high pH, it can be attacked also by *Saccharomonas anaerobia* which renders the product unsaleable. It is not surprising that this disorder is prevalent in France where production is concentrated on naturally sweet ciders of high pH.[21]

Table VI

Effect of centrifuging on the micro-flora of a cider at different stages of fermentation (*o.g.* 1·060)

Specific gravity at centrifuging	Condition	Viable organisms/ml. Yeasts	Viable organisms/ml. Bacteria	Percentage composition of yeast flora *Saccharomyces*	Percentage composition of yeast flora *Torulopsis*	Percentage composition of yeast flora *Kloeckera*
1·055	Before centrifuging	5,900,000	N.D.	61	1	38 (2,250,000)
	After centrifuging	2,155,000	55,000	1	1	95 (2,035,000)
1·050	Before centrifuging	2,050,000	50,000	85	13	2 (5,000)
	After centrifuging	23,000	34,500	100	N.D.	N.D.
1·025	Before centrifuging	2,900,000	50,000			
	After centrifuging	26,500	12,000			
1·000	Racked	260,000	3,250,000			
	Centrifuged	45	1,300,000			
	Pulp filtered	90	50			

N.D. = Not detected

Control methods based on changes in the micro-flora

Pure-culture fermentations

Pure-culture fermentations have often been advocated in view of the dangers of bacterial spoilage when using the methods described above. The cider-maker has problems that in some ways are more difficult than those of the brewer. In brewing, the raw material, malt, can be stored without deterioration and the wort sterilized by boiling. The total capacity of the fermenting vessels forms only a fraction of the annual output (thus they can be constructed of the best materials) and, although the profit margin is small, many hundreds of millions of gallons can be sold because of national demand and the assured outlets of tied houses. On the other hand, the cider-makers' raw material must be processed within three months which means capital equipment cannot be used economically, the capacity of the fermenting and storage vessels must be sufficient for 1 to 1½ year's sales, the factory production of cider is only 22,000,000 gallons and the demand is more seasonal. Consequently pure-culture fermentations are possible only when the selling price of the product can support the extra charges for specialized equipment and additional technical personnel. Thus, champagne cider and perry are successfully produced from depectinized, sterile, filtered or flash-pasteurized juice or diluted concentrates, yeasted with suitable pure cultures and fermented in stainless steel or internally coated vessels. But the selling price of the great bulk of cider is lower, hence the material used for fermentation and storage vessels must be relatively inexpensive. Wood is commonly used, and it is virtually impossible to undertake pure-culture fermentations in vessels of this material.

Dominant fermentations

Pure yeast cultures are widely used on the Continent in another method.[22] It consists in reducing the juice flora by centrifugation or by the addition of small quantities of sulphur dioxide; a large inoculum of yeast is then added and the juice fermented rapidly to 'dryness' at 15°. The process is favoured, since it is said to impart a vinous flavour to what would otherwise be an indifferent cider. The naturally high acidity of the dry ciders is reduced by encouraging a malo-lactic fermentation during storage. Although this method has been used for at least 50 years,[23] there have been no published reports on the fate of the added yeast cultures during fermentation.

In 1951 an investigation was made[6] of the yeast flora of a juice after various pre-treatments and also of the corresponding ciders after yeast additions; the experiments were carried out under normal cider-making conditions, i.e. using wooden fermentation vessels. The effect of the pre-treatment on the micro-flora of the juice is shown in Table VII.

Table VII

Effects of five pre-treatments on the micro-flora of the juice

Juice pretreatment	Viable organisms per 1 ml. Yeasts	Bacteria
Untreated	400,000	200,000
Sulphited (150 p.p.m. SO_2)	6500	1500
Centrifuged	65,000	550,000
Flash pasteurized	245	315
Sterile filtered		
Direct from filter	N.D.	N.D.
In cask	45	5

These results show that sulphiting the juice reduced the total population to 1·25% of that in the untreated sample and was more effective against bacteria than yeasts. On the other hand, centrifuging removed many yeasts but had no such effect on the bacteria.* Flash pasteurization reduced the total population by 99·9%: the machine was run with direct steam injection without any holding period, otherwise a sterile juice would have been obtained. Sterile filtration gave a truly sterile filtrate but the juice was contaminated with organisms from the casks, even though these had been carefully steamed beforehand.

* The apparent increase in the numbers of bacteria in the juice after centrifuging has been noted on other occasions with juices (see Table VI, sp. gr. 1·055) but not with fermenting ciders. It is probably due to the pectinous nature of the juice keeping the bacteria in clumps. Centrifuging would tend to disperse the clumps and so give an apparently higher count. During fermentation the pectin is destroyed by pectic enzymes derived originally from the juice.

Each treated juice was divided amongst three barrels, one of which was left uninoculated; a culture of a cider yeast (probably *Saccharomyces florentinus*) was added to the second, and a wine yeast culture (probably *Saccharomyces steineri*) added to the third. At the end of the fermentations the yeasts in the supernatant ciders and their lees were isolated in pure culture and submitted to a wide range of biochemical and morphological tests which showed certain of these yeasts to be identical with those added originally. The percentages present in the only three ciders in which the culture yeasts survived are given in Table VIII.

Table VIII

Culture yeasts present in the ciders at sp. gr. 1·007
(Expressed as % of the total number of viable yeasts present in each sample)

Yeast	Original juice treatment	Supernatant	Lees
Cider yeast	Sulphited	33	50
Wine yeast	Flash pasteurized	6	38
Wine yeast	Sterile filtered	10	38

Thus, the culture yeasts could be recovered only from ciders made from juices in which the original flora had been greatly reduced; even in these ciders the added yeasts were only partly responsible for the fermentations.

Once fermentation started very few bacteria could be isolated from any cider. None at all were found in the later stages of the sulphited juice fermentations, while in the remaining ciders at sp.gr. 1·007 the bacteria formed less than 1% of the total viable population. The lactobacilli, however, increased enormously in the non-sulphited ciders when samples of all the ciders were left for 6 months on their lees, as shown by the malo-lactic changes given in Table IX.

Table IX

Malic and lactic acid contents of the dry ciders after 6 *months' storage on their lees at* 15°[24]

Juice pre-treatment	Acid composition of dry ciders	
	Malic acid, %	Lactic acid, %
Untreated	<0·01	0·34
Sulphited	0·45	Trace
Centrifuged	0·1	0·30
Flash pasteurized	0·02	0·30
Sterile filtered	0·01	0·33

There was little difference between the acidities of the three ciders from each treatment.

It is obvious that sulphiting the juice is the only method suitable for preventing bacterial changes in factories not equipped for pure-culture fermentations.[25] The addition of pure yeast cultures to

partially sterilized juices does not offer any great advantage in English cider-making, nor does it cause any significant improvement in flavour: ciders from sulphited juices have, in our experience, always had the best flavour.

Sulphited juice fermentations

From the results just given it appeared that sulphiting juices offered a method that would fulfil some of the requirements given at the beginning of this paper; but its effect on the yeast flora was unknown. Earlier work[26] had shown that adding increasing amounts of sulphur dioxide to batches of the same sterile juice, inoculated with a pure yeast culture, led to increased rates of fermentation, once the initial lag phase was overcome (Fig. 2).

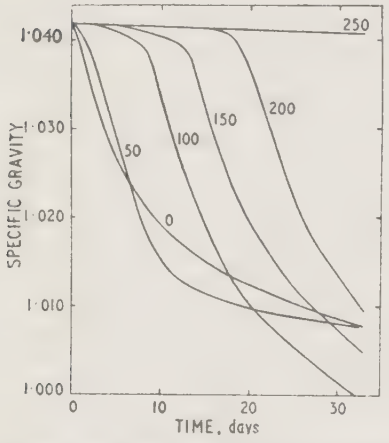

FIG. 2.

The effect of different concentrations of sulphur dioxide on a pure culture fermentation of apple juice.

(Figures on curves are p.p.m. of SO_2)

This pointed to a more efficient yeast fermentation in sulphited juices. It has also been shown[6] that yeasts subcultured in media containing up to 250 p.p.m. of SO_2 increase in cell size, which again would suggest improved powers of fermentation.

Table X shows the changes that take place in the yeast flora of a juice when 150 p.p.m. of SO_2 are added. The treated sample was plated out within two hours of adding the sulphur dioxide. No bacteria were observed in either juice. With the exception of *Candida pulcherrima*, the specific names are provisional at present, but they give an indication of the properties of the yeasts. The flora in the untreated juice is characteristic of that on the fruit and of the majority of juices that have been examined so far. It consists almost entirely of non-sporing yeasts; species of *Saccharomyces* form less than 1% of the total yeast population in the juice and can be isolated only by enrichment techniques. Sulphiting has here reduced the total viable yeasts to 1·3% of the original count and the species that survive have also been greatly reduced in numbers.

Table X

Effect of sulphur dioxide on the yeast flora of a Kingston Black juice

Total viable yeasts	Untreated 160,000	Sulphited 2050
Candida pulcherrima	85,000 (53)	650 (31·5)
C. parapsilosis	15,000 (9·5)	N.D.
C. solani	5000 (3·0)	50 (2·5)
Cryptococcus sp. A.	5000 (3·0)	N.D.
C. luteolus	5000 (3·0)	N.D.
Torulopsis sp. A.	20,000 (12·5)	800 (39)
Torulopsis sp. B.	15,000 (9·5)	550 (27)
Rhodotorula sp.	5000 (3·0)	N.D.
Kloeckera sp.	5000 (3·0)	N.D.

(figures in brackets are % of total)

The changes that occurred during the subsequent fermentations of these two juices are shown in Table XI.

Table XI

Counts of viable yeasts and bacteria during the fermentation of untreated and sulphited Kingston Black juice at 12°.

Stage of fermentation	Untreated juice		Sulphited juice	
	Yeasts	Bacteria	Yeasts	Bacteria
5° drop	12,500,000	N.D.	17,650,000	N.D.
15° drop	22,500,000	N.D.*	37,500,000	N.D.
25° drop	12,750,000	<50,000	25,000,000	N.D.
35° drop	11,300,000	<50,000	6,250,000	N.D.
55° drop (sp. gr. 1·005)	5,800,000	300,000	3,000,000	N.D.

* Aureomycin in the plating out medium, so bacterial counts not possible

These results are not strictly comparable with those obtained on a large scale, since the fermentations were conducted in sterile jars under a layer of sterile liquid paraffin. With this proviso they closely resemble results obtained with the fermentation of other batches of the same untreated juice in wooden barrels (see Table VI). The change from a majority of non-sporing yeasts in the juice to sporing yeasts in the cider is quite dramatic and appears to be valid for all the fermentations examined so far. In these two ciders, all the yeasts resembled *Saccharomyces uvarum*, with the exception of a small number of apiculate yeasts in the early stages of the untreated juice fermentation. There is a greater yeast population for at least the first half of the sulphited juice fermentation, but in both ciders

the numbers of yeasts decrease steadily after this point. A comparison of the change in yeast numbers in the untreated cider with the growth curve shown in Fig. 1 indicates that the viable yeasts tend to decrease considerably in numbers before any appreciable autolysis takes place.

Another feature seen from Table XI is the absence of bacteria in the sulphited juice fermentation, in contrast to the increase in the numbers of bacteria in the natural fermentation as the viable yeast count declines. The juice used for this experiment was of medium pH (3·6) which tends to restrict bacterial activity. Bacterial changes occur most rapidly above pH 4·0 and it has been shown that a malo-lactic change can occur in juices of this pH, even before fermentation begins.[20] Table XII shows that the bacterial population of a cider from a juice with high pH can be affected markedly by sulphiting the fresh juice.

Table XII

Viable yeast and bacteria in dry ciders made from a juice of pH 4·2

	Viable count/ml.	
	Yeasts	Bacteria
Dry cider from untreated juice	100,000	4,300,000
Dry cider from the same juice treated with 100 p.p.m. of SO_2	170,000	< 23,000
Dry cider from the same juice treated 200 with p.p.m. of SO_2	680,000	N.D.

Thus, sulphiting can restrict the numbers of lactic acid bacteria even of ciders made from juices of high pH.[27] It follows, therefore, that the rate of fermentation of such sulphited ciders can be regulated safely by centrifuging, since the numbers of bacteria in the centrifugate will be negligible. Only *Saccharomonas anaerobia* is unaffected by this treatment, being capable of growing in the presence of 500 p.p.m. of SO_2,[28] but growth can occur in sulphited juices of high pH and low nitrogen content if they are centrifuged to make naturally sweet ciders: such ciders can be protected against this disorder only by reducing the pH to below 3·7.

Sulphiting the juice as a method of yeast control is now used for experimental fermentations and in the industry. A cleaner and fresher flavoured cider is produced, and oxidized tannin hazes in high-pH ciders are minimized so that filtration is easier. There are still some problems to be solved. Occasionally a malo-lactic or malo-succinic change occurs during the fermentation of sulphited juices of high pH in which sulphur dioxide is known to be least effective:[29] these changes are not accounted for solely by pH. Again, the acidity of ciders from sulphited juice often increases

during fermentation by as much as 30% due to the production of malic acid;[27] whether this is due to yeast or bacterial activity is not yet known. The lack of taints in the aroma and flavour of sulphited juice ciders is also interesting, since it may be due either to reduction in the non-sporing yeasts in the juice or to the elimination of some species of anaerobic bacteria not yet isolated.

Conclusions

It has been shown that variability in the fruit samples can influence the course of the subsequent fermentations, and so no one single control method can be adopted universally. The circumstances in which the different methods should be employed may be summarized as follows:

(1) Pure-culture fermentations are satisfactory when suitable plant and technical personnel are available and the value of the cider is sufficient to cover the additional costs.

(2) Juices with low pH can be allowed to ferment naturally under anaerobic conditions, since the pH will prevent undesirable bacterial changes. On prolonged storage, malo-lactic fermentation will gradually reduce the acidity sufficiently to make a palatable cider.

(3) Juices with medium and high pH should always be sulphited and then fermented anaerobically.

(4) Centrifugation is suitable for reducing excessively fast rates of fermentation in non-sulphited juices of low pH or sulphited juices of medium or high pH.

(5) Excessively slow rates of fermentation can be corrected by adding soluble nitrogen and/or thiamine in amounts just sufficient to allow fermentation to 'dryness'.

(6) Naturally sweet ciders should be made only from juices of medium or low pH otherwise they are liable to be infected by *Saccharomonas anaerobia*.

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Discussion

Mr. A. E. Wiles: (1) *S. uvarum* was mentioned as the predominant yeast during the main fermentation. Is this organism considered to be the one mainly responsible for cider fermentations in various parts of England?

(2) One is struck by the low yeast counts during fermentation (as compared with brewing) apart from the obvious speed-up in fermentation; what other effects would arise from adding yeast to such levels?

(3) If pure culture is usually uneconomic, could not yeast be purchased?

Mr. Beech: (1) In the small number of juices examined so far *Saccharomyces uvarum* has been the predominant yeast. A much larger range of juices must be examined before it can be stated as being the predominant yeast in cider fermentations. Its presence has been noted also by Osterwalder in Switzerland and by Capitain in France from fermenting pear juices.

(2) If heavy concentrations of yeast are added to apple juice the fermentation is violent and completed within one or two weeks. Ciders from such fermentations have little character in their aroma and flavour. A similar effect is found in wine making; thus the finest wines are prevented from fermenting too rapidly.

(3) Pure-culture fermentations are not used since the selling price of the product does not support the extra costs necessary for sterilizing the juice and the purchase of suitable fermentation and storage vessels. The addition of, e.g., brewer's yeast would not suppress the spoilage organisms derived originally from the juice.

Mrs. Tritton: When you mentioned soluble nitrogen, did you mean formol nitrogen or total nitrogen?

Mr Beech: The term soluble nitrogen refers to the Kjeldahl estimations of nitrogen on a filtered sample of juice or cider. Thus it represents the nitrogen content of the amino-acids, amides, purines, pyrimidines and inorganic nitrogenous salts.

Dr. H. Joergensen: Will 200 p.p.m. of SO_2 be allowed by the authorities in Great Britain and Ireland?

Mr. Beech: 200 p.p.m. total sulphur dioxide is the maximum permissible amount allowed in the finished cider in Great Britain.

Mr. J. J. O'Leary: You mention that you use pH adjustment for control. How do you adjust the pH and at what stage is the adjustment carried out?

Mr. Beech: We endeavour to adjust the pH of the juice by blending juices of high and low pH. If all juices are low in pH then they are not adjusted further since the pH protects the cider against unwanted bacteriological changes during fermentation. The pH of the cider is then raised during storage by encouraging a malo-lactic fermentation, or in extreme cases by adding small quantities of potassium carbonate.

If most of the juices are of high pH, addition of SO_2 causes the formation of malic acid and a decrease in pH. If the pH is still too high when ready for sale, it is reduced further by the addition of citric acid.

The object of making these changes are threefold, (*a*) to control the development of lactobacilli, (*b*) to suppress *Saccharomonas anaerobia*, which cannot grow below pH 3·7, and (*c*) to produce a cider with a pleasant balance of acidity.

Dr. C. Rainbow: Do the lactobacilli associated with cider fermentations show (1) similar nutritional fastidiousness as dairy and brewing lactobacilli with respect to growth factors, amino-acids and purine and pyrimidine bases? (2) unusual pH–growth relationships?

Mr. Beech: (1) The cider lactobacilli have nutritional requirements similar to lactobacilli isolated from beer (J. G. Carr, 1956, Doctoral Thesis, University of Bristol). (2) Growth only occurs below pH 6·5. The optimum is between pH 4 and 5 but some growth can occur slowly at pH 3·2 – 3·3.

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THIRD SESSION

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Chairman: DR. A. K. MILLS

REGULATION OF CARBOHYDRATE METABOLISM IN YEAST CELLS

By HELMUT HOLZER*
(*University of Hamburg*)

In this paper are discussed two enzymically regulated alterations in the carbohydrate metabolism of living cells of baker's yeast: (1) the occurrence of aerobic fermentation in connection with growth, and (2) the inhibition of glucose uptake under transition from anaerobic to aerobic conditions known as the Pasteur effect.

Growth and aerobic fermentation

The correlation between growth and aerobic fermentation in baker's yeast cells is demonstrated very clearly when ammonium salts are added to glucose-oxidizing yeast, which could not grow because of lack of nitrogen-containing salts. As is shown in Fig. 1, intensive aerobic fermentation commences soon after the

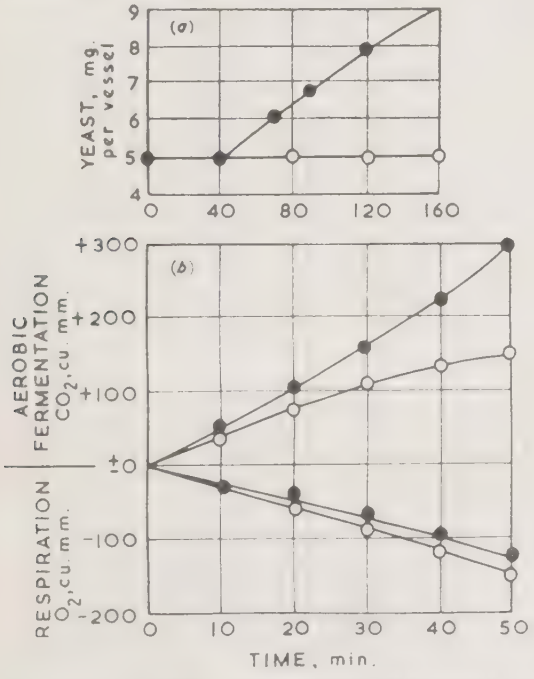

FIG. 1. *Respiration, aerobic fermentation and growth at 30° after addition of $(NH_4)_2SO_4$ to glucose-oxidizing yeast cells.*[16]

(*a*) • with $(NH_4)_2SO_4$ o without $(NH_4)_2SO_4$ (*b*) • 1·3% glucose + 0·01 M-$(NH_4)_2SO_4$; o 1·3% glucose alone (5 mg. of baker's yeast per vessel.)

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addition of ammonium ions. After an induction period of about 40 min., growth and cell division begin, which can be measured turbidimetrically. These experiments were done with high concentrations of ammonium ions — about 0·01M.

Fig. 2 demonstrates that small amounts of ammonium ion cause only a small additional aerobic fermentation. There is a production of about 6–8 moles of carbon dioxide per mole of added ammonium ion by additional aerobic fermentation.

The initiation of aerobic fermentation after the addition of ammonium salts is explained by the following theoretical considerations which were shown to be valid by experimental work. According

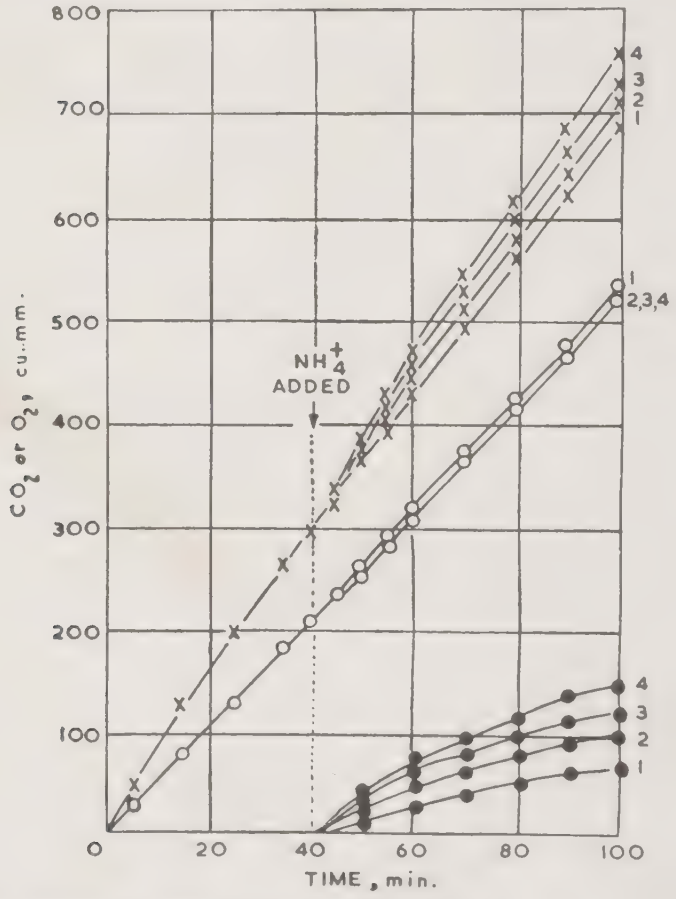

FIG. 2. *Action of small amounts of ammonium ions on aerobic fermentation*[5]
1 without NH_4^+; 2 with 0·2 μM-NH_4^+; 3 with 0·4μM-NH_4^+; 4 with 0·6μM-NH_4^+

× $+CO_2$ o $-O_2$ • aerobic fermentation $= CO_2 - O_2$

to Warburg & Christian[1] and Bücher[2] the rate of triosephosphate dehydrogenation in a cell-free system depends on the concentrations of inorganic phosphate and ADP*:

phosphoglyceraldehyde + phosphate + ADP + DPN ⇌
phosphoglyceric acid + ATP + DPNH.

Fig. 3 shows the effect of phosphate on this reaction as determined by Warburg with crystallized triosephosphate dehydrogenase.

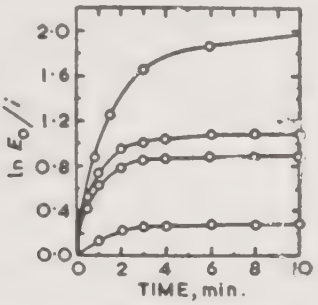

FIG. 3. *Rate of triosephosphate dehydrogenation with different concentrations of orthophosphate*[1]

I (bottom curve) $0{\cdot}87 \times 10^{-6}$ M-phosphate/ml., pH 7·4; II $1{\cdot}68 \times 10^{-5}$ M-phosphate/ml., pH 7·4; III $3{\cdot}36 \times 10^{-5}$ M-phosphate/ml., pH 7·4; IV (top curve) $3{\cdot}36 \times 10^{-5}$ M-phosphate/ml., pH 8·45

The curves I–III show that increase in the initial concentration of phosphate increases the initial rate of the triosephosphate dehydrogenase reaction.

According to Lynen,[3] in glucose-oxidizing yeast cells most of the phosphate of the adenylic acid system is present in the form of ATP. This is due to the fact that during the oxidation of one mole of glucose up to 36 moles of ATP can be formed and only 2 moles of ATP are required for the phosphorylation initiating the breakdown of 1 mole of glucose. Therefore the rate of glucose metabolism depends on the velocity with which ATP is split into ADP and P, the necessary components of the triosephosphate dehydrogenase reaction.

In Fig. 4 this restriction on the glucose metabolism of yeast cells is shown in a schematic presentation of carbohydrate degradation. From this figure it is seen that a more rapid formation of P and ADP from ATP must accelerate the breakdown of triosephosphate.

With this background, the effect of ammonium ions on aerobic fermentation is now discussed. Glucose-oxidizing yeast, which cannot grow because of lack of nitrogen, requires only very small amounts

* Abbreviations used: P = inorganic phosphate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; DPN = diphosphopyridine nucleotid; CoA = coenzyme A.

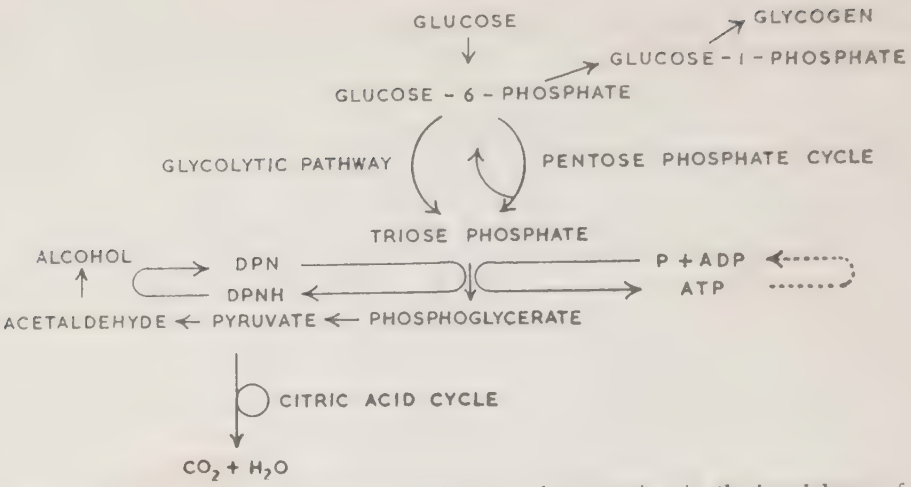

FIG. 4. *Triosephosphate dehydrogenation as a key reaction in the breakdown of carbohydrate*

of ATP. Consequently the concentration of P and ADP is also small, since only a small amount of the ATP synthesized is used up. P and ADP are just sufficient for glucose oxidation and in addition only a very small aerobic fermentation can take place.

On addition of ammonia the synthesis of amino-acids, proteins, pyrimidines and other nitrogen-containing substances will take place. For these syntheses ATP is necessary, which means that P and ADP are released.

energy-delivering dissimilatory processes

$$\text{ATP} \xleftarrow{\qquad} \text{ADP} + \text{P}$$

energy-consuming synthetic processes

The excess of ADP and P resulting from the incorporation of nitrogen makes an accelerated dehydrogenation of triosephosphate possible, which in turn leads to aerobic fermentation, i.e., an additional formation of alcohol, since the capacity of oxygen-consuming oxidative enzyme systems was already taxed to the limit before addition of ammonium ions. This is made evident by the fact that the addition of NH_4^+ does not produce any increase in O_2-uptake in the manometric test, as shown in Figs. 1 and 2.

If these considerations are correct, the release of 6–8 moles of CO_2 per mole of NH_4^+ (see Fig. 2) would mean that 1 mole of NH_4^+ leads to the consumption of 6–8 moles of ATP and therefore to a release of 6–8 moles of P and ADP. This seems to be due to the fact that 6–8 ATP-requiring steps are involved in the incorporation of nitrogen into the end products of nitrogen assimilation (e.g., proteins, nucleic acids, etc.).

The above considerations concerning the relations between ATP consumed, ADP and P release, triosephosphate dehydrogenation, aerobic fermentation and growth, which have been discussed are theoretical. The results of the following experiments present evidence that these considerations are correct with respect to yeast cells.

Fig. 4 demonstrates the common pathway of fermentation and oxidation down to the formation of pyruvic acid. From this point on the two processes are different. In fermentation, pyruvate is decarboxylated to acetaldehyde, while in the oxidative pathway it is oxidized to acetyl-CoA:

glucose→pyruvate ↗ acetyl-coenzyme A→citric acid cycle
glucose→pyruvate ↘ acetaldehyde→alcohol

If the hypothesis stated is correct, i.e., that oxidative processes first utilize the pyruvate produced in the dehydrogenation of triosephosphate and that only the excess of pyruvate can be converted into acetaldehyde and alcohol, then pyruvate oxidase should have a greater affinity for pyruvate than has carboxylase. Fig. 5 shows that this is so. Purified carboxylase, isolated from yeast maceration juice, has a Michaelis constant of 10^{-3} moles per litre, determined by an optical test using a solution of the carboxylase with alcohol dehydrogenase and DPNH. Pyruvate oxidase is localized in yeast mitochondria and has Michaelis constant of 10^{-4} moles per litre,[4] as determined by measurement of the colour change of *p*-nitroaniline during *N*-acetylation. The addition of liver transacetylase is

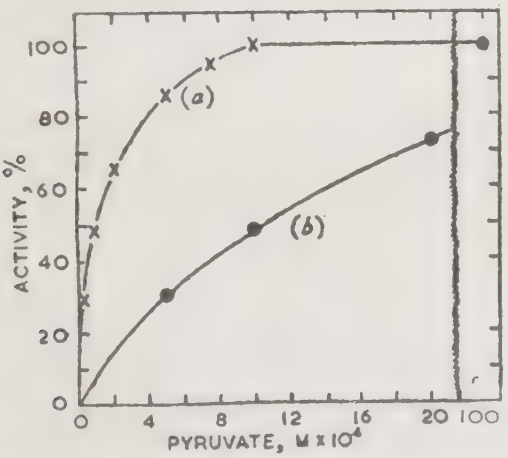

FIG. 5. *Affinity of pyruvate decarboxylase and pyruvate oxidase to pyruvate*[4]
curve (*a*) pyruvate⁻ + DPN⁺ + CoA→acetyl - CoA + DPNH + CO_2
curve (*b*) pyruvate→ acetaldehyde + HCO_3^-

necessary to transfer the acetyl group from coenzyme A to nitroaniline. Coenzymes in the oxidative system are magnesium, thiamine pyrophosphate, DPN and coenzyme A. Addition of thioctic acid produced no further activation of the oxidase. It seems probable that in the intact mitochondria the enzyme is already saturated with thioctic acid.

Table I demonstrates the increasing pyruvate and the decreasing α-ketoglutarate level after the addition of ammonium salt to glucose-oxidizing yeast.[5] Both effects agree completely with the theory proposed. The result of activated triosephosphate dehydrogenation is an accumulation of pyruvate which leads to aerobic fermentation by activation of carboxylase. As Trevelyan *et al.*[6] clearly demonstrated, the rate of fermentation in living yeast cells depends indeed on the pyruvate concentration inside the cells. α-Ketoglutarate decreases because after addition of NH_4^+ there is an intensive reductive amination to glutamic acid. The presence of the DPN specific enzyme required for this reaction in animal tissues has been known for a long time. It has also been demonstrated that it is present in high concentration in baker's yeast as well as in brewer's yeast.[7] Table II shows the results of a purification procedure for glutamic dehydrogenase from brewer's yeast. At present an attempt is being made to characterize the enzyme more precisely with respect to coenzyme requirement, Michaelis constant, equilibrium constant, etc.

Table I

Steady-state concentrations of pyruvate and α-ketoglutarate in glucose-oxidizing yeast cells with and without ammonium sulphate[5]

	Aerobic without $(NH_4)_2SO_4$	Aerobic with $(NH_4)_2SO_4$
Pyruvate, μmoles/g. of cells ...	3·4	6·2
α-Ketoglutarate, μmoles/g of cells	3·7	0·6
Pyruvate, μmoles/ml. of medium	0·06	0·06
α-Ketoglutarate, μmoles/ml. of medium	0·16	0·02

Recently the existence of another enzyme was demonstrated in brewer's yeast which is interesting in this connexion, namely a transaminase which transfers the amino-group of glutamic acid—the formation of which has already been discussed—to other α-keto-acids. This enzyme is most active in the reaction

glutamate + oxaloacetate ⇌ α-ketoglutarate + aspartate.

Table II

Purification of a DPN-specific glutamic acid dehydrogenase from brewer's yeast[7]

Purification step	Total units	Specific activity, units/mg. of protein
(1) Lebedev juice	$3{\cdot}2 \times 10^5$	31
(2) Fraction precipitated on addition of ammonium sulphate to 35% concentration	$2{\cdot}9 \times 10^5$	157
(3) Precipitate from (2) dissolved in water, treated with Al $(OH)_3$ gel and eluted with phosphate buffer solution pH7·4 ...	$2{\cdot}0 \times 10^5$	605

Table III demonstrates the results of the purification of the transaminase as worked out by Gerlach & Jacobi.[8] Fig. 6 shows that yeast transaminase, like the corresponding animal and bacterial enzymes, requires pyridoxal phosphate as coenzyme. The Michaelis

Table III

Purification of a transaminating enzyme from brewer's yeast[8]

Purification step	Total units	Specific activity (units per mg. of protein)
(1) Lebedev juice	94×10^4	138
(2) Lebedev juice after heating at 60°	61×10^4	264
(3) Precipitated from solution (2) after addition of ammonium sulphate to 22—27% concentration, dialysed against water	28×10^4	388
(4) Dialysate from (3) treated with $Al(OH)_3$ gel, and eluted with buffer solution ...	6×10^4	941

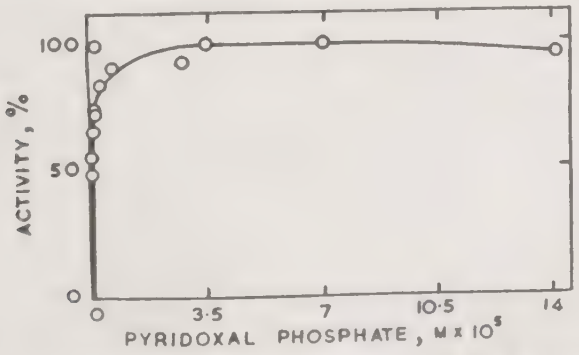

FIG. 6. *Requirement of the yeast transaminase for pyridoxal phosphate*[8]

constant is about 10^{-7} moles per litre. Investigations on substrate specificity and other features of the enzyme are in progress.

From what is known at present, ammonium ion is incorporated essentially with the aid of the DPN-specific glutamic acid dehydrogenase described above by reductive amination of α-ketoglutarate (besides this the formation of carbamylphosphate might be a second reaction by which ammonium ion is taken up). If this is correct, one should find a decreasing steady-state concentration of DPNH after the addition of ammonia. This was proved to be true in experiments done with Lynen.[9] As is seen from Table IV the quotient acetaldehyde/alcohol increases by 2–3 times after addition of ammonia. Since it was shown that in glucose-oxidizing yeast cells

Table IV

Calculation of the quotient DPNH/DPN from determinations of alcohol and acetaldehyde in glucose oxidizing living yeast cells[9]

	aerobic	aerobic in presence of NH
acetaldehyde:alcohol... ...	1:76	1:30
free DPNH:freeDPN... ...	1:1100	1:3000

the alcohol dehydrogenase equilibrium is always maintained as a result of the high activity of alcohol dehydrogenase, an increase of the quotient acetaldehyde/alcohol implies a diminution of the quotient DPNH/DPN:

$$K = \frac{\text{alcohol}}{\text{acetaldehyde}} \times \frac{\text{DPN}}{\text{DPNH}}$$

Thus the steady-state concentration of DPNH has decreased, as expected from theoretical considerations.

Finally, the optical test developed by Bücher[10] for the determination of ATP enabled us to prove the expected decrease of ATP after the addition of ammonia, while inorganic P and ADP increased. Fig. 7 illustrates an experiment carried out by Holzer & Witt.[11] In the figure is seen the very rapid decrease of α-ketoglutarate after addition of ammonia, which is evidence for the ammonia fixation via α-ketoglutarate amination.

There are therefore considerable data to support the hypothesis dealing with the enzymically regulated relations between aerobic fermentation and growth. These relations in chronological order are summarized in Fig. 8.

Immediately after the addition of ammonium salts a reductive amination of α-ketoglutarate begins. Therefore the concentration of DPNH falls and the quotient acetaldehyde/alcohol increases.

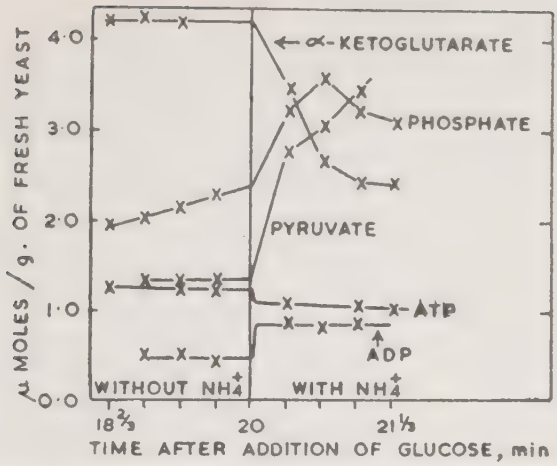

FIG. 7. *Steady-state concentrations of several metabolites in glucose-oxidizing yeast cells before and after addition of ammonium sulphate*[11]

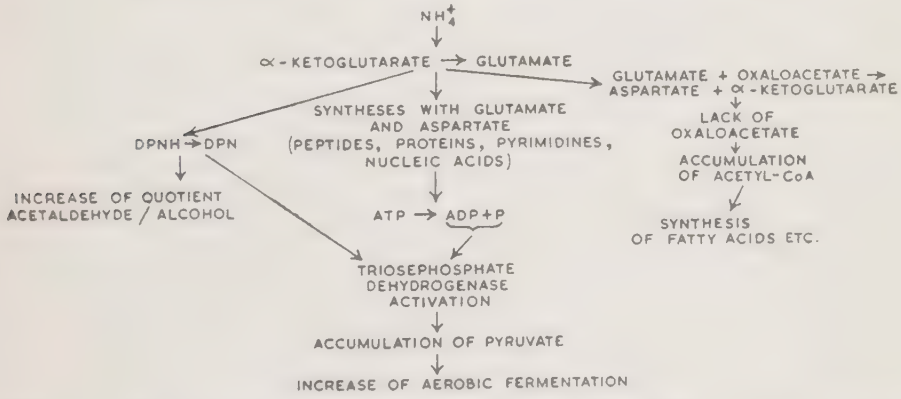
NH4+
α-KETOGLUTARATE → GLUTAMATE
GLUTAMATE + OXALOACETATE → ASPARTATE + α-KETOGLUTARATE
LACK OF OXALOACETATE
ACCUMULATION OF ACETYL-CoA
SYNTHESIS OF FATTY ACIDS ETC.
SYNTHESES WITH GLUTAMATE AND ASPARTATE (PEPTIDES, PROTEINS, PYRIMIDINES, NUCLEIC ACIDS)
DPNH → DPN
INCREASE OF QUOTIENT ACETALDEHYDE / ALCOHOL
ATP → ADP+P
TRIOSEPHOSPHATE DEHYDROGENASE ACTIVATION
ACCUMULATION OF PYRUVATE
INCREASE OF AEROBIC FERMENTATION

FIG. 8. *Chronological order of the events after the addition of ammonium sulphate to glucose-oxidizing yeast cells*

The increase in the concentration of glutamic acid leads to an increased formation of aspartic acid by transamination. Both amino-acids facilitate the synthesis of other amino-acids by transamination and therefore the synthesis of peptides and proteins. Furthermore, after the addition of ammonium salts the synthesis of carbamylphosphate from ATP, CO_2 and ammonia begins. The carbamylphosphate is a precursor of pyrimidines. All these ATP-requiring reactions increase the concentration of ADP and P and therefore accelerate the dehydrogenation of triosephosphate. As a result of this, more glucose is metabolized and pyruvate accumulates. Thus the decarboxylation of pyruvate is activated and more acetaldehyde and alcohol will be formed, i.e., there is an increase in aerobic fermentation.

It will be interesting to analyse more metabolites on the pathway which leads from α-ketoglutarate and pyruvate to the complex pool of cell material synthesized during growth. Such studies may give further information on the interplay of enzymes during growth. A similar situation occurs in animal cells. Warburg[12] pointed out the close connexion between malignant growth and increased aerobic glycolysis.

Pasteur effect

The Pasteur effect consists of the decrease of glucose metabolism in yeast when anaerobic conditions change into aerobic. Fig. 9 demonstrates this effect in an experiment utilizing modern methods.[13] To explain the phenomenon several theories have been proposed, the most important of which are discussed below.

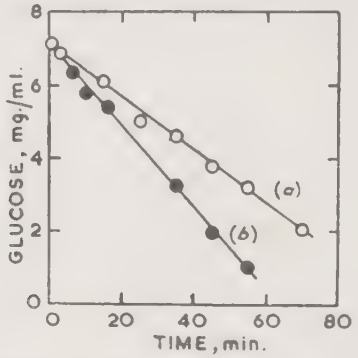

FIG. 9. *Glucose uptake of living yeast cells under aerobic and anaerobic conditions* (Pasteur effect)[13]

(*a*) aerobic (*b*) anaerobic

According to Lynen[3] and Johnson[14] the enzyme triosephosphate dehydrogenase is responsible for the retardation of glucose metabolism by oxygen. It is well known that an intensive ATP synthesis starts within the mitochondria when changing from anaerobic to aerobic conditions:

$$P + ADP \rightarrow ATP$$

Therefore within the cells inorganic phosphate as well as ADP must decrease. This in return retards triosephosphate dehydrogenation and thereby glucose metabolism (the dependence of triosephosphate dehydrogenation on phosphate and ADP has already been demonstrated in Figs. 3 and 4.)

Fig. 10 presents an experiment done by Lynen,[3] which shows that the concentration of inorganic phosphate actually does decrease when glucose-metabolizing yeast cells are transferred from anaerobic to aerobic conditions.

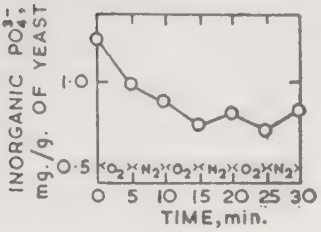

FIG. 10. *Steady-state concentrations of phosphate in living yeast cells under transition from aerobic (O_2) to anaerobic (N_2) conditions and vice versa*[3]

The theory of Lynen and Johnson is supported by the experiments presented and it is assumed that the phosphate-regulating mechanism does work in living yeast. But as pointed out by Lynen in 1951,[13,15] there is an experimental finding, which cannot be explained by this theory, and therefore an additional mechanism must exist. The problem is this: when under aerobic conditions the concentration of ATP has increased, one should expect an increased rate in the irreversible hexokinase reaction:

glucose+ATP→glucose-6-phosphate+ADP

This implies that under aerobic conditions more glucose should be phosphorylated and disappear from the medium than under anaerobic conditions. Now the Pasteur effect states the exact contrary, as has been shown in Fig. 9: under aerobic conditions less glucose disappears than under anaerobic conditions. The results of an experiment described in Fig. 11 make it possible to explain this difficulty. This figure indicates that the ATP concentration does not increase but remains the same when changing from anaerobic conditions. Because of the unchanged concentration of ATP, it might be thought that the Pasteur effect has not commenced, but this is not so because all the other known changes in concentrations

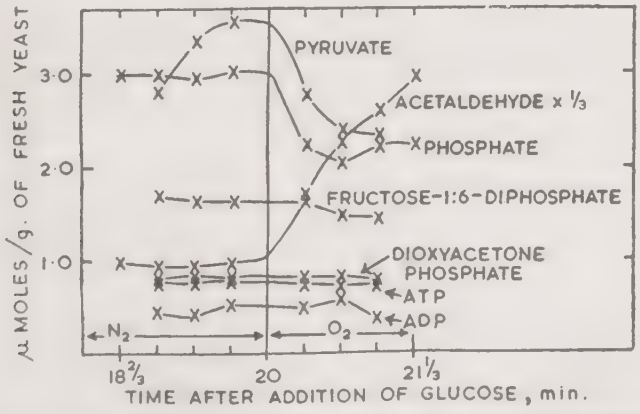

FIG. 11. *Steady-state concentrations of ATP and other metabolites in living yeast cells under transition from anaerobic to aerobic conditions*[17]

of metabolites have taken place: inorganic phosphate is decreasing,[3] acetaldehyde is increasing,[5,9] and pyruvic acid decreases.[16] In accordance with the ideas of Lynen[15] the explanation is that, in the aerobic phase, ATP is indeed increasing at the place of its additional formation, namely in the mitochondria, but in the cytoplasm its concentration decreases. This leads to the total constant amount of ATP in cytoplasm plus mitochondria which was found in the experiment illustrated by Fig. 11. This explains why in changing to aerobic conditions the enzyme hexokinase phosphorylates less glucose, because this enzyme is localized in the cytoplasm where it has to work with less ATP. The intracellular dislocation of ATP together with the hypothesis of Lynen and Johnson offers a complete explanation of the mechanism of the Pasteur effect. Nevertheless it will be necessary to prove by further direct experimental work that ATP actually does move from the cytoplasm into the mitochondria under aerobic conditions.

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Discussion

Dr. A. K. Mills: What is the relationship between free and bound DPN and DPNH? Could you give us some more information about the turnover of pyrophosphate under aerobic conditions?

Dr. Holzer: (1) Free and bound DPN and DPNH: according to experiments of Warburg and Velick, DPN and DPNH are bound to yeast alcohol dehydrogenase with different dissociation constants. Since the concentration of alcohol dehydrogenase in living yeast cells is extremely high, a great deal of the total DPN and DPNH is bound to the protein and cannot exist freely diffusing in the cytoplasm. As

the dissociation constant for DPNH is ten-fold smaller than for DPN, the quotient DPNH/DPN for the bound pyridine nucleotides is very different from that for the free pyridine nucleotides. We could demonstrate that in glucose-metabolizing yeast cells the mentioned quotient is for the free DPNH/DPN about 1:1000 and for the total DPNH/DPN (sum of free plus bound nucleotides) about 1:5 (Holzer, Schultz & Lynen, *Biochem. Z.*, 1956, **328**, 252,). Since the free nucleotides only are thermodynamically active in enzyme systems it will be necessary for energy considerations to calculate using the concentrations of free nucleotides and not as hitherto usually done with the total concentrations. (2) Turnover of pyrophosphate: the following enzymically catalysed reactions for instance must be taken into account to explain the participation of pyrophosphate (PP) in yeast metabolism:

DPN+PP=nicotinamide mononucleotide+ATP

acetyl-coenzyme A+PP+AMP=acetate+ATP+coenzyme A.

Prof. E. J. Conway: I should like to know what views Dr. Holzer has concerning the origin of α-ketoglutaric acid in the yeast cell. It could readily function as the precursor of succinic acid from the experiments of Kleinzeller, but, so far as I can understand, Prof. Krebs does not support the view that the tricarboxylic acid cycle is functional in yeast.

Dr. Holzer: Prof. Krebs believes that the enzymes of the tricarboxylic acid cycle in yeast have not sufficient activity to allow of the *complete* respiration (oxygen uptake with glucose) in the process of the cycle, but according to Prof. Lynen there is evidence that the complete respiration does follow the pathway of the cycle. It might be that some enzymes of the cycle (malic dehydrogenase and *iso*citric dehydrogenase), for which Prof. Krebs found only a small activity in baker's yeast, are extracted with difficulty from the cell structure, and therefore the observed activities are much smaller than the true activities. Further experimental work will be necessary to prove this possibility.

MEASUREMENTS OF LEVELS OF METABOLITES WITHIN THE YEAST CELL DURING FERMENTATION

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Introduction

Recent work on living yeast has shown that the reactions of the Embden–Meyerhof cycle (shown in a simplified form in Fig. 1) by which sugars are fermented through a series of intermediates to pyruvic acid, and thence anaerobically to ethanol and carbon dioxide, or in the presence of oxygen to carbon dioxide and water in the Krebs cycle, constitute the main system for metabolising carbohydrate in yeast.[1] Other systems, for example, the oxidative shunt operating from glucose-6-phosphate, are of relatively minor importance, and may serve only as a synthetic route for pentoses and their derivatives. The fermentation enzymes are freely suspended in the yeast cell, and unlike the respiration enzymes, are not separated by a selectively permeable membrane in the mitochondria. It is possible, however, that the coenzymes that are common to the fermentation and respiration systems are not homogeneously distributed.

Enzymologists have provided a large mass of data on the *in vitro* properties of the individual purified enzymes of the fermentation cycle, which often include the Michaelis constants, the dissociation constants of the enzyme–substrate complexes and the equilibrium constants of the reactions controlled. In addition, estimates of the amounts of the enzymes present in various sources and the turnover

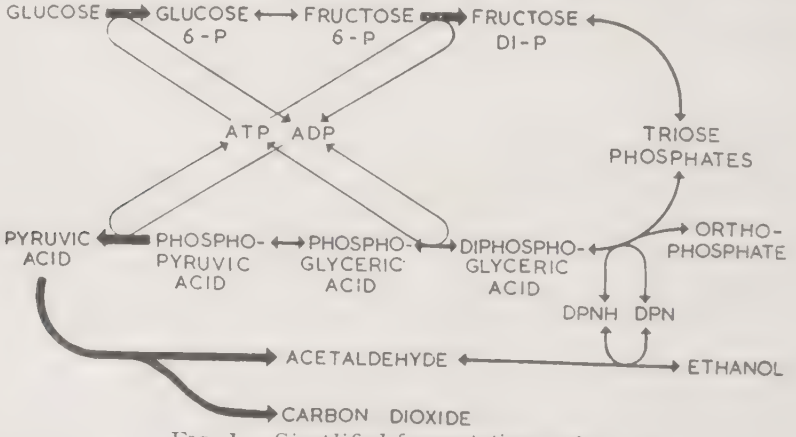


FIG. 1. *Simplified fermentation cycle*
6P = 6-phosphate. di-P = diphosphate.

numbers of the enzymes are available in some cases. It is apparent that, in yeast, certain enzymes, notably triose phosphate dehydrogenase, aldolase and triose isomerase, are present in relatively large amounts, and these enzymes all catalyse reversible reactions. As a consequence, the intermediates that are linked by such reversible reactions would be expected to be in equilibrium at the steady state of fermentation, a concept that can be verified by appropriate measurements of intermediates during fermentation. The fermentation cycle is thus considerably simplified and can be considered as a series of groups of intermediates in equilibrium, the groups separated by effectively irreversible reactions. The fermentation coenzymes occupy a key position in the scheme and can regulate the rate of fermentation by delicate interactions from one point in the series to another. For example, in the irreversible reactions between glucose and pyruvic acid which are all linked to the adenosine coenzymes, the effect of any local factor that influences the rate of one of these reactions is transmitted to all the others by changes in the coenzyme levels so that balance is restored. Similarly, the rate of the irreversible decarboxylation of pyruvate is linked to the previous reactions through the subsequent reduction with diphosphopyridine nucleotide to ethanol. The rate of the irreversible reactions will depend on the concentrations of both the substrate intermediates and the coenzymes, but not on the products of the reactions. When the over-all rate of fermentation is altered, the concentrations of these intermediates and coenzymes will be readjusted until all the irreversible reactions are working at the same rate, and a new steady-state balance is restored. When, however, an enzyme is saturated with respect to both its substrates, an absolute limitation on the over-all rate of fermentation is imposed. Now, it is believed that certain baker's yeasts studied have such an absolute limitation when fermenting in simple glucose medium, and this is caused by the saturation of carboxylase.[2]

This paper describes attempts made in these laboratories to add to our understanding of the regulation mechanism of fermentation by providing information about the levels of a range of fermentation intermediates and other metabolites, including coenzymes and related nucleotides, in living yeast which is actively fermenting under various conditions. In particular the interest was in the way in which the patterns of these fermentation intermediates and coenzymes alter in relation to different conditions that affect the rate of fermentation. Some consideration of the conditions of growth, which in all probablility influence the composition of the enzymes, is clearly desirable. While it is most probable that the same factors

will influence the regulation of fermentation in all samples of yeast, the relative importance of these factors may differ from yeast to yeast, giving different concentration patterns for the intermediates. Such an investigation can only be effectively conducted in a comprehensive manner, since a large amount of data is required from each fermentation in order to characterise its regulation mechanism.

Separations by anion-exchange chromatography

For separating intermediates and nucleotides and for unambiguous determination of many compounds on such a scale, ion-exchange chromatography appeared to be more suitable than other techniques such as paper chromatography or electrophoresis.

The intermediates of fermentation are essentially ionic materials; acidic properties in many cases have been conferred on them by the presence of phosphate groups. Nucleotides, which are the ribosides of certain purine and pyrimidine bases with one, two or three phosphate groups, are similarly acid. In addition to being units of the important nucleic acids, they are of interest as coenzymes of many metabolic reactions, including fermentation. The pioneer work of Cohn[3] and his associates in America has shown that the adenosine, guanosine, uridine and cytidine nucleotides have different affinities for the strongly basic anion-exchange resins made from polystyrene substituted with quaternary ammonium groups, and the nucleotides, when adsorbed on columns of these resins, can be removed in a definite order by solvents of suitable ionic concentration and pH. The sugar phosphates and a number of organic acids that are important metabolites, such as pyruvic, ketoglutaric and succinic acids, can be separated by similar cycles of adsorption and elution on the same resins. For example, hexose, pentose and nucleotide diphosphates are eluted subsequent to the corresponding monophosphates. This relation between structure and affinity has been extended somewhat, and some regularity is observed between the affinity of an acid for a resin, as measured by its distribution between the resin and the solvent at equilibrium, and the dissociation constant of the acid. The coefficients calculated for a number of acids from such distribution experiments are also measures of the volumes of solvent required to elute them under comparable conditions, a fact that is sometimes useful in choosing the elution conditions for the optimum degree of separation.

The task of choosing a suitable routine procedure was complicated by the fact that metabolites of a wider range of types than had been separated previously by a single ion-exchange procedure were involved, at least in the initial stages of the work. For example, the high chromatographic resolution of nucleotides obtained from

extracts of various tissues and from yeast by Potter and his co-workers[4] using a formate cycle (that is, adsorbing on a resin that has all its basic groups combined with formate, and eluting the adsorbed acidic materials in a definite sequence with a series of formic acid and formate solvents) were most impressive. Unfortunately the high concentration of formate in these eluates interferes seriously with the analyses of fermentation intermediates, especially the sugar phosphates, and for general use we have preferred to operate anion-exchange columns on the chloride cycle.

Fig. 2 shows typical chromatographic separations of nucleotides in extracts of resting yeast by chloride solvents at pH 2 and pH 5. (In Fig. 2A Eluent 1 is 0·001 → 0·01 N-HCl; Eluent 2 is 0·01 N-HCl + 0 → 0·45 M-NaCl; Eluent 3 is 0·45 M-NaCl; and Eluent 4 is N-HCl. In Fig. 2B the eluent is 0 → 0·5 M-NH_4Cl.) The chloride ion has a much higher replacement power than formate, so that similar, if slightly less sharp, separations can be obtained with solutions of much lower concentration, and, for example, hexose phosphates can be estimated directly on the eluates with the anthrone reagent. In addition, compounds in the eluates can be rechromatographed on another column after adjusting the pH and diluting until the total anion concentration does not exceed 0·01 N. The dilution required with most formate eluates makes this direct procedure prohibitive. The order of elution of the nucleotides differs at the two pH, and it has been shown that compounds which are eluted together at pH 2 can often be completely separated by rechromatography with elution at pH 5.[4] This two-column technique is analogous in its resolving powers to two-dimensional paper chromatography.

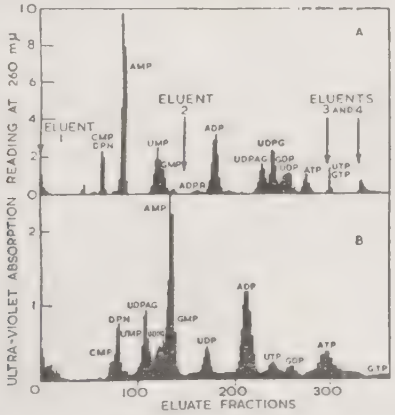


FIG. 2. *Anion-exchange chromatography of extracts of resting yeasts*
A eluent HCl and HCl/NaCl solutions pH2
B eluent ammoniun chloride solutions pH5

For abbreviations see page 84.

Arrangements were made for the simultaneous chromatography of up to eight yeast extracts on a battery of exactly similar ion-exchange columns (0·3 sq. cm. cross-sectional area, filled to a depth of 42 cm. with Dowex-1-chloride, 10% cross-linked, 200–400 mesh), so that strict comparisons could be made, either between a series of extracts taken from different stages of a fermentation, or between a number of different fermentations. This comparative approach to ion exchange does not appear to have been generally attempted, but it is essential both to avoid introducing errors from variations in yeast performance at widely separated intervals, and also to maintain a reasonable rate of progress, since a single chromatographic run occupies a period of some two weeks. In order to keep the time for a single set of comparative experiments down even to this period an automatic fraction-collecting apparatus was designed,

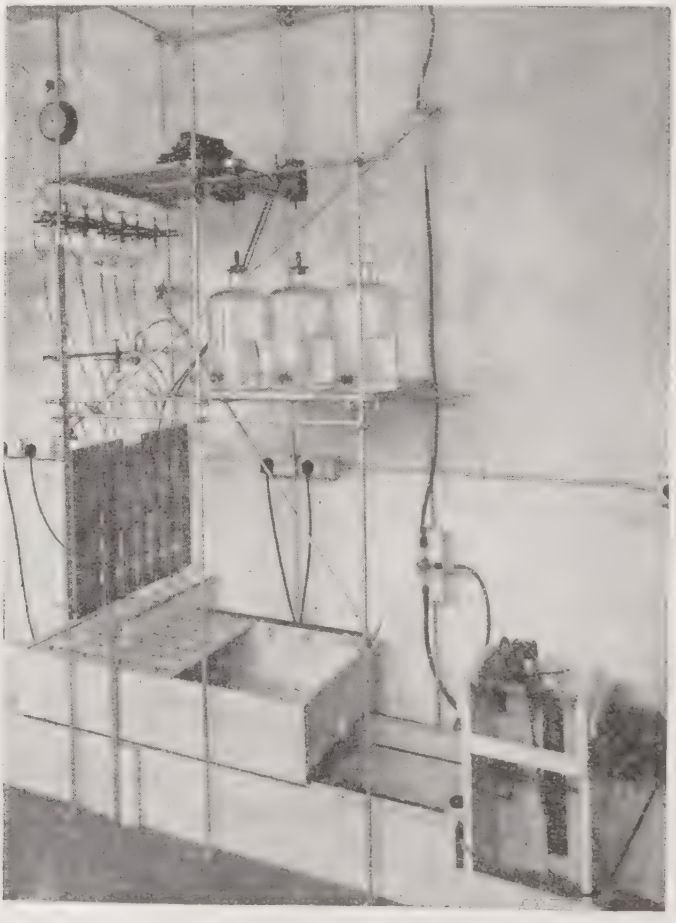


FIG. 3 *Apparatus for ion-exchange chromatography showing columns, elution system and fraction cutter*

operating on the time-flow principle, that could deliver eluates from eight columns simultaneously into rows of tubes. This apparatus (Fig. 3) could be adjusted to various time intervals for collecting eluates, and since it could operate for 64 hours without attention, collecting some 40 fractions from each column in that time, the elution could proceed continuously night and day until complete.

Normally about two hundred fractions are collected from each column and thus altogether nearly two thousand fractions from a single run require analysis. For a typical routine separation from an extract of fermenting yeast as shown in Fig. 4, rapid analyses were made by spectrophotometric measurements, especially at a wavelength of 260 mμ, where all the nucleotides absorb strongly. The progress of the elution can thus be followed with reasonable rapidity, and other analyses for compounds that do not absorb ultra-violet light can be carried out on the minimum number of fractions at the appropriate points in the elution. Thus, the hexose phosphates can be measured with the anthrone reagent, using the direct heat-of-mixing technique, the fructose phosphates being distinguished from the glucose phosphates by using two reagents with different strengths of sulphuric acid.[5] Other non-hexosic intermediates, such as phosphoglyceric acid and phosphopyruvic acid, are estimated by total-phosphate measurements in appropriate fractions.

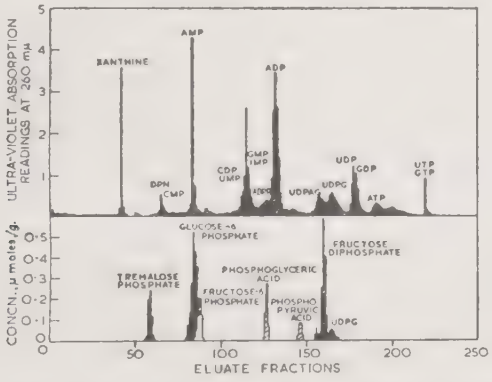


FIG. 4 *Anion-exchange chromatography of extract of yeast fermenting glucose with phosphate*

■ total hexose □ fructose ▒ total phosphate

The validity of relying on such general methods of analysis in routine fractionations is based on both comprehensive and specific data obtained with similar fractions from many previous runs, and in particular from single-column runs (Fig. 5) where a much wider range of analyses has been performed, and results checked against

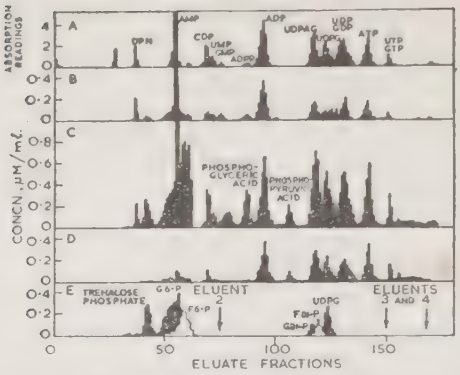


FIG. 5 *Anion-exchange chromatography of extract of fermenting yeast*

A Ultra-violet absorption at 260 mμ
B Pentose C Organic phosphate
D Acid-hydrolysable phosphate E hexose
■ glucose □ fructose

other published chromatographic results where they were available. The type of nucleotide causing a peak in the ultra-violet absorption at 260 mμ, can be determined by carrying out measurements at other wavelengths and in acid and alkaline solution, since the different types all have characteristic spectra which vary differently with pH. (The eluents of Figs. 4 and 5 are the same as for Fig. 2A).

Preparation of yeast extracts

The difficulties of obtaining a yeast extract that accurately represents the concentration of intermediates inside the yeast cell are well known. The heat-treatment method for disrupting the yeast cell was preferred, since this appears to be quicker in operation than perchloric acid, and avoids the high acidity and anion concentration associated with trichloroacetic acid. There is, however, a danger that heat-stable enzymes (e.g. myokinase) may still be active in the extracts. The present method, which is novel, involves passing the yeast suspension from the fermentation apparatus through a glass coil (200 cm. × 0·2 cm. diam.) which is immersed in a bath of boiling water, at a suitable speed (100 ml./min.) so that the temperature is raised in a few seconds to within a degree or two of that of the bath. The hot suspension is kept for a few minutes before being cooled to room temperature, and the dead cells are centrifuged off. The extract is then deproteinized by shaking with amyl alcohol and chloroform,[6] and the solvent layer, with precipitated proteins, is separated by centrifugation. The extract, after adjusting the pH, usually to pH 8, is then ready for passing through an anion-exchange column.

Gradient elution

Eight similar columns, loaded with anions from eight yeast extracts that are being compared, are eluted simultaneously in parallel with solvent from a common elution system. Now it is common experience that, although a solvent of constant composition can be chosen to give the best separation of two compounds with different affinities for an anion-exchange resin, such a solvent cannot be used for separating a range of compounds without either telescoping the earlier compounds together, or widely spacing the later compounds, which are then obtained at very great dilution. It is usual, therefore, to change the solvent periodically, preferably at a point where no compound is being eluted. When a large number of compounds of similar affinities are being separated, as in the chromatography of yeast extract, it is frequently difficult to find a point at which to change the solvent without interfering with at least one compound being eluted. Moreover, it would be necessary to follow the course of the elution very closely, in a manner not practical for continuous operation. Extensive use has therefore been made of the methods of gradient elution, by which the concentration of the eluate is steadily increased as elution proceeds. The methods usually employed involve running a more concentrated solution from one vessel into a weaker solution in another vessel, which is stirred and the mixture, the concentration of which gradually approaches the concentration of the stronger solution, is used for elution.[7–9] This arrangement (Fig. 2A) is often used, but it only partially solves the problem since the rate of change of concentration gets less and less, and soon a more concentrated solvent must be provided.[4] One simple improvement published by Parr[10] is illustrated in Fig. 6B. It can be shown that the rate of change of concentration obtained from this system is linear. This two-pot linear system has been used for the initial stages of the chloride elutions, where it is necessary to increase the acidity up to 0·01 N-HCl (e.g., Eluent 1 in Fig. 2A, and Fig. 5). Subsequently the acidity is kept constant, and increases in chloride concentration are obtained with sodium chloride (e.g. Eluent 2 in Fig. 2A and Fig. 5). For this stage a three-pot system was devised (Fig. 6C), in which the second and third pots contain the weaker solution. Here the rate of change of concentration varies as the square of the volume used. Occasionally a complete fractionation of yeast extract at pH 5 has been carried out with ammonium chloride, and, since there is no acidification stage here, the entire elution has been affected with a three-pot elution system, the compounds in the eluates being reasonably distributed through the fractions (Fig. 2B).

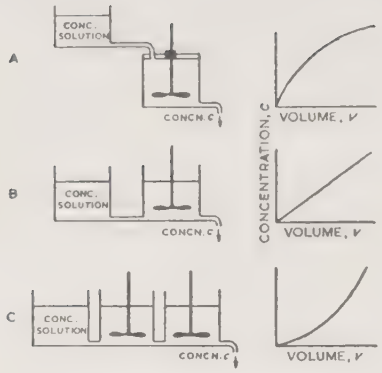


FIG. 6 *Systems for gradient elutions*

A $C \propto 1 - e^{-V}$

B $C \propto V$

C $C \propto V^2$

Conditions influencing chromatographic separation

In the early stages of this ion-exchange study a number of experiments were made to determine the effect of factors influencing the separation of metabolites on anion-exchange columns. As mentioned above, the position of a compound in the eluates depends on its affinity for the resin, that is, on its distribution coefficient at equilibrium. The sharpness with which compounds are eluted, and hence the degree of separation obtained is, however, dependent on other factors such as the length of the chromatographic column, and on factors that determine how near the conditions on the column are to equilibrium, such as rate of flow, size of resin particles, and the degree of cross-linking of the resin.[11] When gradient elution is used, the conditions of elution are constantly changing and an infinite number of distribution equilibria are set up for each compound. It has been shown theoretically that an elution gradient reduces "tailing" and should therefore increase resolution,[7] but clearly, if the concentration of eluate has changed appreciably during the time a single compound is eluted, there will be danger of contamination from the next compound eluted at a slightly higher concentration.

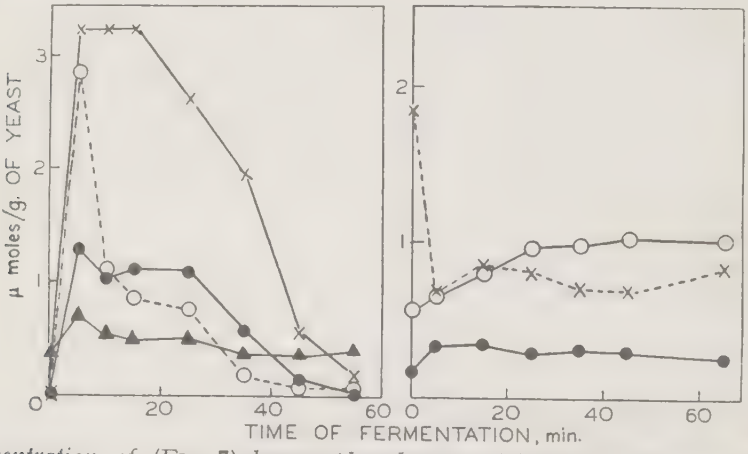
It is of importance to obtain the best possible separation of compounds within a reasonable time, for, provided two compounds adjacent in the order of elution are more or less completely separated and unambiguously identified, the quantitative determination of either can be based on a non-specific method of analysis, for example, on single spectrophotometric measurements or on general hexose estimations. When the overlap is small it is possible to determine the proportions of each in the mixed fractions from considerations of symmetry, provided that the shape of the elution–concentration curve approximates to the normal curve of error, that is, provided that the flow rate is sufficiently slow to enable equilibrium conditions to be established in the column.[11] When, however, the overlap is

considerable, one or both of the compounds must be analysed either by a specific method, as in the analysis of fructose phosphate in the presence of glucose phosphate using a specific anthrone reagent, or in the analysis of two mixed known nucleotides by spectrophotometric measurements at two or more suitable wavelengths. Apart from the inconvenience of extra analyses, determinations based on differences are less accurate and the purity of mixtures is less easy to check. Unfortunately the main factors that improve resolution, such as extra column length, slower flow rates, and lower elution gradients, necessarily involve the elution taking a longer time, and in practice improvements caused by altering one set of conditions must be offset by altering others adversely. Thus, a compromise must be sought, and in general it has been preferred to use slow, near-equilibrium flow rates (0·5 ml./min./sq. cm.) and reasonably long columns (42 cm.). Consequently the rate of increase of the concentration of eluate in relation to the eluate volume has been high. Since the total volumes of eluates are small under these conditions, the metabolites are obtained in high concentration, which is a factor that aids accurate analyses, and enables the loading of extract on the columns to be low. The chromatographic separations achieved have been satisfactory for a general survey of metabolites, and the procedures are sufficiently flexible to allow modifications to suit future needs.

Anaerobic fermentations with small amounts of glucose

In earlier experiments fermentations of small amounts of glucose (initially 0·05 M) were carried out with 5% suspensions of yeast without buffers or other additions. The glucose fermented away completely in about 50 min. at 30°, and so it was possible, by continuous sampling and heat-extraction, to obtain a complete range of extracts for examination by anion-exchange chromatography which corresponded to all the stages of the fermentation. The concentration of the fermentation intermediates, including the hexose monophosphates and fructose diphosphate (Fig. 7) rapidly rose to a maximum and fell away as fermentation proceeded, and finally the available glucose ran out. It is interesting that, even with excess sugar, the maximum rate of fermentation is only maintained for a short time, and the early fall in the maximum level of intermediates is probably not due to the lowering of the glucose concentration. Similar curves have been obtained for pyruvic, phosphopyruvic and phosphoglyceric acids, and thus the intermediates, that on theoretical grounds based on the approximate equilibrium positions can be expected to be present in measurable quantities, have been isolated, with the exception of the triose phosphates.

There is evidence, based on alkali-labile phosphate determinations, for the presence of triose phosphates in the fermenting extracts, but measurable quantities of these intermediates have not been identified. The very rapid rise and fall in the level of trehalose phosphate that occurs in the early stages of fermentation (Fig. 7) may be connected with the dramatic changes in the concentration of the reserve carbohydrate trehalose in yeast which also occurs,[12] since trehalose phosphate is believed to be an intermediate between trehalose and the other hexose esters of the glycolytic cycle. Uridine diphosphoglucose, which may also be involved in carbohydrate metabolism, similarly shows a high concentration at this early stage of fermentation (Fig. 7). In the four nucleotide systems studied, those of adenosine (Fig. 8), uridine, guanosine and cytidine, phosphorylation that accompanies fermentation takes essentially a similar course, possibly on account of transphosphorylation reactions.



Concentration of (Fig. 7) *hexose phosphates and* (Fig. 8) *adenosine phosphate in yeast during fermentation of glucose*

Fig. 7
X hexose monophosphates
O trehalose phosphate
● fructose diphosphate
▼ UDPG

Fig. 8
X AMP
O ADP
● ATP

In the resting condition in all cases the monophosphates are present in higher concentrations than the di- or triphosphates, but at an early stage in fermentation, probably before the maximum rate is attained, the diphosphate concentrations become greater than those of the monophosphates. Proportionate increases in the triphosphates also occur, but the actual amounts of the triphosphates present are usually less than a third of the corresponding diphosphates.

Fermentations by different strains of baker's yeast

Comparisons were made between the changes in level of intermediates and nucleotides during fermentation by a number of samples of baker's yeast which had been grown under similar conditions from different strains of *Saccharomyces cerevisiae*, and which had similar rates of fermentation in plain glucose medium. With the single exception of pyruvate, the levels of intermediates and nucleotides, and their behaviour during fermentation, were not very different in the various yeast samples.

Anaerobic and aerobic fermentations with excess glucose

In more recent experiments the data have been reinforced from anion-exchange chromatography by making a number of other measurements. The approximate rate at which glucose disappears from fermenting suspensions has been calculated in order to estimate the rate at which the enzymes of the glycolytic cycle deal with their substrates. The rate of production both of carbon dioxide and ethanol was measured, so that the proportion of anaerobic and aerobic metabolism can be estimated from the difference. In addition, the intracellular levels of the key fermentation intermediate, pyruvic acid, were determined from the differences between measurements of pyruvate in the supernatant liquor and in extracts from the fermentations. In these experiments, instead of studying the course of a single fermentation in each chromatographic run, the characteristics of a number of different fermentations were compared, taking a single extract for chromatography from each.

A special all-glass apparatus was constructed for carrying out these fermentations on a one-litre scale. It was equipped with internal glass coils through which water at 30° was passed to control the fermentation temperature, and had a built-in glass sinter at the base for gassing the fermentation either with nitrogen or air. The carbon dioxide evolved was absorbed from the effluent gas stream during definite periods of time by passing through scrubbing towers containing glass beads covered with alkali, and estimated after precipitation as barium carbonate.[13] The fermentations were carried on for nearly an hour before they were stopped by passing through the heated coil (mentioned under Preparation of yeast extract). The yeast concentration was lowered to 2·5%, and the glucose concentration was raised to 0·5 M, so that the fermentation should not be limited by the glucose concentration.

In one set of fermentations the effect was investigated of a number of levels of added orthophosphate up to 60 μmoles/g. of yeast to otherwise plain glucose fermentations, gassed with both nitrogen and air. All the amounts of added phosphate were taken up by the

yeast during the fermentation period: it should be mentioned in this connexion that the yeast samples that we have studied were grown in a medium with limited phosphate. The rate of carbon dioxide evolution in all fermentations rose to a maximum in about 20 min. and then declined somewhat. Fig. 9 shows the average rate of production of carbon dioxide and ethanol. The effect of different levels of added phosphate was progressively to cause a slight increase in the rate of fermentation. The effect of passing air through the fermentation instead of nitrogen was to reduce the rate of carbon dioxide production by about a quarter. Anaerobically the rates of carbon dioxide and ethanol production were almost equivalent; in the aerated fermentation the ethanol production was less than a half that of carbon dioxide. If it is assumed that the ethanol is a measure of the rate of anaerobic fermentation, then the proportion of aerobic metabolism can be calculated from the difference. The amount of aerobic metabolism was only about one-third of that taking place anaerobically at the same time, and thus the aerated fermentations were only partially aerobic. The total rate of metabolism was about half that of the fully anaerobic fermentation, a fact that was roughly confirmed by sugar uptake measurements.

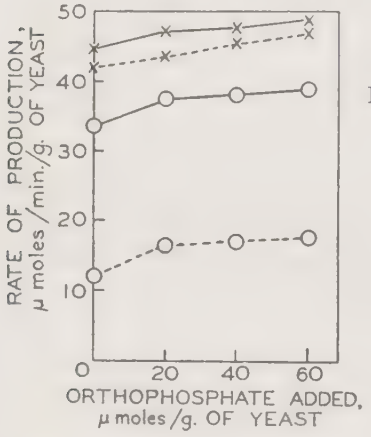
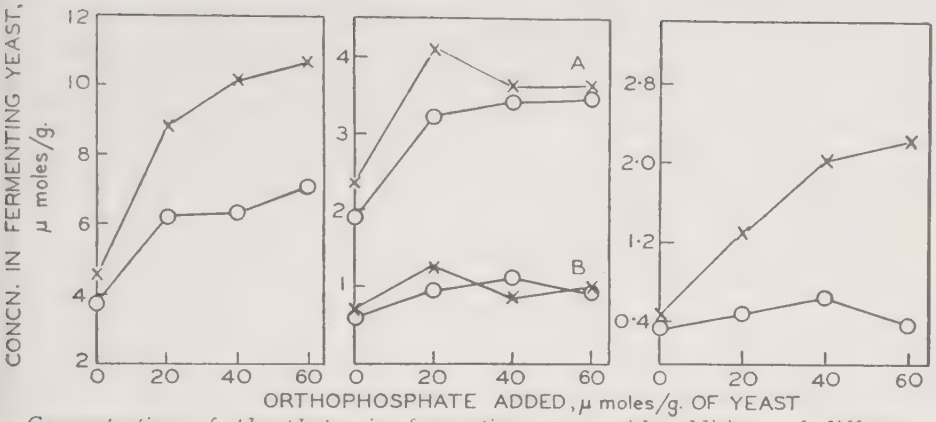


FIG. 9 *Average rate of production of CO_2 and ethanol during* 0–40 *min. fermentation with excess of glucose and different amounts of orthophosphates*

x——x CO_2 anaerobic
x - - - x ethanol anaerobic
o——o CO_2 aerobic
o - - - o ethanol aerobic

The effect of adding orthophosphate to anaerobic and aerobic fermentations on the internal orthophosphate, on the fermentation intermediates and on the adenosine coenzymes, are shown in Figs. 10–15. The increase in the concentration of these metabolites became less as greater quantities were assimilated, and the remainder of the assimilated phosphate must have been stored away in other compounds such as metaphosphate and nucleic acids. The rate of fermentation was increased by a relatively modest amount (Fig. 9), especially when compared with the large increase in the fructose diphosphate. It thus appears that the assimilated orthophosphate

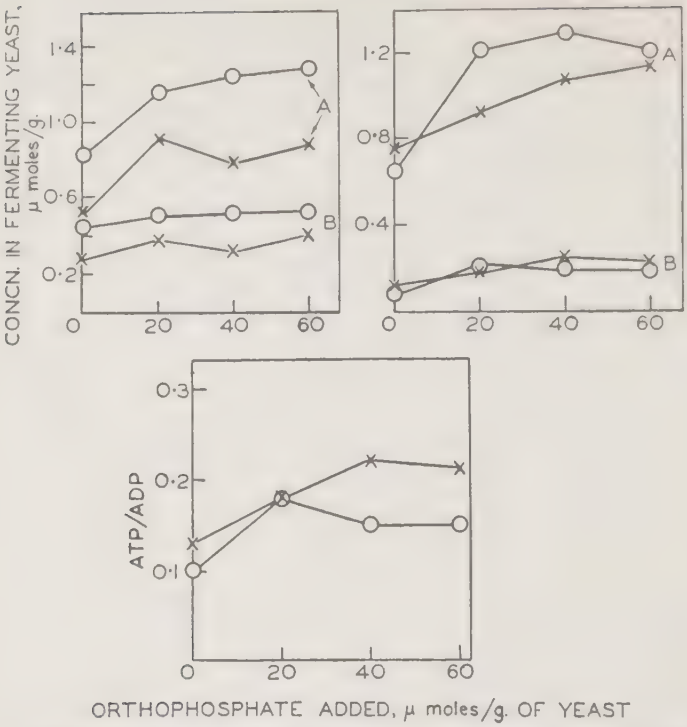


Concentration of phosphates in fermenting yeast with additions of different amounts of orthophosphate

FIG. 10 (left) Orthophosphate FIG. 11 A glucose 6-phosphate B fructose 6-phosphate FIG. 12 (right) Fructose diphosphates
X anaerobic O aerobic

had been distributed among the metabolites with least interference with the rate of fermentation; or conversely, the yeast could ferment in the absence of nitrogen and other growth materials at essentially the same rate in spite of large differences in the available phosphate. In accordance with Le Chatelier's principle, natural selection has endowed the yeast cell with the ability to oppose, by suitable internal rearrangements, the constraints of Nature, which take the form of changes in available phosphate and other food supplies, and also changes in physical conditions such as temperature or osmotic pressure. It is necessary for the cells to be able to regulate the rate at which they use up food supplies, both those external to the cells and those laid down as cell carbohydrate, and, as a direct consequence, to be able to provide an ordered pattern of intermediates at appropriate concentrations so that synthesis of cell materials may proceed smoothly. Therefore, for example, different levels of internal orthophosphate, or of the ratio of adenosine triphosphate to diphosphate under different conditions, may be considered not so much as the rate-limiting factors in fermentation but as necessary adjustments that the yeast cells have made in these factors in order to compensate for the change in conditions, and to restore a regular pattern of intermediates.

The small effect of aeration on the levels of adenosine triphosphate (Figs. 14 and 15) were unexpected in view of the much greater number of moles of this coenzyme generated per mole of sugar metabolized under aerobic conditions. It has been suggested, however, that this excess adenosine triphosphate is bound up in the



Concentration of intermediates in fermenting yeast with additions of different amounts of orthophosphates

FIG. 13 (top left) A phosphoglyceric acid B phosphopyruvic acid FIG. 14 A ADP B ATP

FIG. 15 (below) Ratio ATP/ADP
X anaerobic O aerobic

mitochondria and is thus not available to the fermentation enzymes. Figs. 16 and 17 show that the data are in agreement with the hypothesis that the enzymes which catalyse the reversible reactions of the glycolytic cycle are in excess, even at different velocities of fermentation, and so cannot regulate these velocities. As a consequence, the intermediates that are linked by such reversible reactions would be expected to be in equilibrium with each other. This is supported by the constancy of the ratios of fructose monophosphate to glucose monophosphate, and of phosphopyruvic acid to phosphoglyceric acid in all the experiments which involved a range of individual values. The series of reactions linking fructose diphosphate to phosphoglyceric acid are complicated by the participation of orthophosphate, the oxidized and reduced forms of diphosphopyridine nucleotide and adenosine di- and tri-phosphates. The two forms of diphosphopyridine nucleotide unfortunately were not measured, but assuming that the ratio between them is unity and

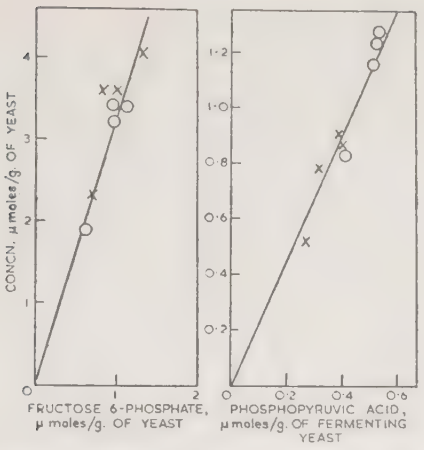


FIG. 16 (left) *Relation between glucose* 6*-phosphate and fructose* 6*-phosphate in fermenting yeast*

FIG. 17 *Relation between phosphoglyceric and pyruvic acids in fermenting yeast*

X anaerobic O aerobic

making due allowance for the accumulation of errors, the data are consistent with fructose diphosphate and phosphoglyceric acid being in equilibrium. Under aerobic conditions this equilibrium is shifted markedly in favour of phosphoglyceric acid, presumably by an increase in the ratio of the oxidized to the reduced forms of the coenzyme. The remarkable effect of added phosphate in increasing the fructose diphosphate concentration can now be understood since, owing to the dismutation of fructose diphosphate into the two triose phosphates, it is the square root of the concentration that effects the equilibrium position.

Rate limitation by carboxylase

Separate fermentation experiments on the yeasts used in this investigation have shown that the carboxylase system imposes a limitation on the rate of fermentation in media deficient in thiamine. In a complete medium containing thiamine, the maximum rate of fermentation is greatly increased and the intracellular level of pyruvate, the substrate for carboxylase, falls. The addition of phosphate to both the anaerobic and the aerated fermentations in plain glucose medium increases the levels of pyruvate, the effect of phosphate being greater on increasing the concentration of intermediates in front of pyruvate than on increasing the rate of fermentation. In the aerated fermentations the pyruvate levels fall, since the pyruvate is being partially metabolized by aerobic pathways not involving carboxylase.

Concluding remarks

This paper is considered merely as a preliminary contribution to a complex problem. The patterns of the intermediates need to be studied under many other fermentation conditions, including those in which the carboxylase system is not saturated, and the experimental procedures must be both broadened in scope and refined in detail. However valuable may be the data provided by enzymologists on isolated enzyme systems, the interpretation of these data in terms of the living cell must be the aim of biochemists, and this integration can probably be profitably attempted even at this stage.

Abbreviations used

CMP = cytidine monophosphate
AMP, ADP, ATP = adenosine mono-, di-, and tri-phosphates
UMP, UDP, UTP = uridine mono-, di- and tri-phosphates
GMP, GDP, GTP = guanosine mono-, di- and tri-phosphates
DPN = diphosphopyridine nucleotide
ADPR = adenosine diphosphate ribose
UDPG = uridine diphospho-glucose
UDPAG = uridine diphospho-acetylglucosamine

Acknowledgments

The authors wish to thank their colleagues for much useful discussion, Miss M. E. Moorse, Messrs. J. K. Fadden and K. G Harnden for assistance with the experiments, and the Directors of the Distillers Company, Limited for permission to publish this paper.

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Discussion

Dr. A. A. Eddy: Does one encounter a mannose analogue of UDP glucose in the extracts you described?

Mr. Mann: The only mannose-nucleotide encountered in the extracts was the well-known guanosine diphospho-mannose compound.

Dr. C. Rainbow: What are your conditions for the determination of 'easily hydrolysable phosphate'?

Mr. Mann: The 'easily hydrolysable phosphate' was determined by heating the solution for 10 minutes in N-H_2SO_4 at 100°.

Mr. J. S. Harrison: A composite dynamic picture of the response of yeast to various changes in environment is presented, in an admittedly over-simplified attempt (based on a four-dimensional imagination), to unify various ideas put forward at the Symposium. Although microscopically the yeast cell appears as a cell-wall (glucose and mannose units, Prof. Jørgensen, and carrying an electric charge, Dr. Eddy), glycogen particles (glucose units), nucleus (nitrogenous), mitochondria and possibly fat (Dr. Challinor) and metaphosphate (Dr. Holzer and Mr. Mann), the transparent aqueous phase is highly important, containing hundreds of different molecular species, including the enzymes of the fermentation cycle (Mr. Mann), in free 'solution'. Supposing a sequence of glucose molecules enters the cell at a given point and follows the fermentation cycle (illustrated by Dr. Holzer and Mr. Mann), then addition of thiamine may accelerate the carboxylase action (Mr. Mann) and changes must inevitably occur indirectly at all points throughout the cycle, due to an alteration in the rate of formation of co-enzymes which interact on one or more reactions.

Similarly the effects of the addition of phosphate, oxygen and assimilable nitrogen to glucose (referred to as P O N G) are to change the pattern of intermediate levels by different pathways, and as a result to alter the rates of certain reactions.

The assimilable nitrogen, in particular, initiates many synthetic reactions by which the yeast substance is increased, and within some hours the yeast has doubled in all its individual components. These processes continue in the presence of adequate supplies of nutrients – as an example, to the theoretical extent of 2^{300} times in one of Dr. Slonimski's experiments. Using a conservative estimate of mutation rate of 1 in 2^{10}, this would produce (again theoretically) 2^{290} mutants with their many problems for the geneticist and cytologist.

ACTIVE TRANSPORT IN THE YEAST CELL

By Prof. E. J. CONWAY
(*University College, Dublin*)

Introduction

The growing or metabolizing cell in general requires material from its surroundings, which enters the cell by passive diffusion or is actively transported. The entrance of such material into the yeast cell occurs during fermentation and is almost entirely effected and controlled by active transport.

It is considered in this paper that active transport of a solute is necessarily associated with free-energy transfer from another system and involves a carrier mechanism. Passive diffusion across a membrane is differentiated from active transport essentially on this energy question but may occur in various ways such as:
(*a*) through molecular pores, (*b*) by solution in the membrane and diffusion therefrom towards an equivalence of chemical or electrochemical potential, (*c*) by carrier adsorption and exchange without free-energy donation (e.g., the 'exchange diffusion' of Ussing[1]) or (*d*) by the passage of individual solute molecules or ions beyond a certain statistical velocity, making their way, as it were, through a barrier.

In active transport the basic considerations are the nature of the carrier system, the way in which energy is donated thereto and the manner in which such energy is utilized.

Various aspects of the transport in yeast have been reviewed in articles by different authors and read at the Congress on Active Transport of Ions and Molecules held at Bangor (1953) and published by the Society of Experimental Biology.[2] Recent reviews of special interest are those of Rothstein[3] (who also deals with metabolic processes such as stages in glycolysis) and of Harris.[4]

Permeability of cell walls

Before dealing with the nature of active transport in yeast, some features of the external surface require consideration. There is an outer metabolic chamber of the yeast cell which may be identified with the yeast cell wall.[5] The water for solution in this outer chamber is 0·11 of the total cell volume. Into this chamber many substances enter freely, including potassium and sodium chlorides, also various sugars such as glucose, galactose and arabinose. Inulin and peptone do not enter and such an ion as succinate enters only slowly.

With respect to the passive permeability, the inner surface of the outer chamber in resting anaerobic yeast is practically impermeable

to K^+, Na^+, Cl^- and phosphate and other inorganic ions, but labelled K^+ will exchange freely with the internal K^+ on shaking with oxygen.

Besides the permeability to such substances, the permeability to certain organic acids is important for the interpretation of various points connected with active transport, glycolytic stages, etc. Thus Table I shows the ratio of total 0·2M organic acid which has entered 1 kg. of yeast cells after various times.

It will be seen that a number of organic acids including succinic, glyceric, pyruvic, citric, etc., and amino-acids such as glycine, leucine, glutamic acid, etc., have entered the yeast cell (apart from the outer chamber) very slowly or at no measurable ratio over 60 min., whereas formic, acetic, propronic and butyric acids enter fully or almost so in 15 min. Also, as may be expected, substances of high lipid solubility enter very rapidly.

Besides such permeability there is the fact that the outer surface of the yeast cell is in general negatively charged because of fixed anions, a fact which has been dealt with by Eddy (see p. 3–5 above).

Table I

Ratio of total acid that has entered 1 kg. of yeast to the external resulting concentration (the R value)

Organic acid	*R* value After 15 min.	*R* value After 60 min.	*R* value After 1440 min.	pH of mixture after 60 min.
Succinic	0·11	0·14	0·24	2·9
Glyceric	0·15	0·15	0·11	2·8
Pyruvic	0·16	0·20	—	3·0
Malic	0·26	0·27	0·42	2·6
Fumaric	—	0·25	0·96	2·9
Lactic	—	0·19	1·44	3·2
Citric	0·11	0·10	—	2·3
Tartaric	0·20	0·22	—	—
Gluconic	—	0·24	—	2·6
Oxalic	0·06	0·08	—	3·0
Glycine	0·08	0·11	—	3·0
Alanine	—	0·15	—	2·9
Leucine	0·14	0·08	—	2·8
Aspartic	0·15	—	—	3·2
Glutamic	0·06	0·06	—	3·6
Valine	—	0·23	—	2·7
Formic	1·75	1·75	—	3·2
Acetic	1·71	1·67	1·70	3·3
Propionic	1·30	1·50	—	3·7
Butyric	1·30	1·50	—	3·6

The uptake of glucose

Glucose (and other monosaccharides) enters the outer chamber of the yeast cell readily and is then, like the other readily fermentable monosaccharides phosphorylated in the yeast cell wall or at the site

of the lipid membrane. This phosphorylation is carried out by the hexokinase system, and the process or uptake appears very similar to that occurring with cells, muscle fibres and the like in animal tissues. Galactose and arabinose are not ordinarily taken up by yeast in appreciable amounts apart from their entrance into the outer region. Maltose is also readily fermentable by yeast and, although this may be assumed to occur after hydrolysis to glucose prior to the action of the hexokinase system, there is evidence that it can occasionally be fermented at a faster rate than glucose and may thus be directly phosphorylated.

Many reviews of carbohydrate metabolism in its various stages have appeared. In Rothstein's review,[3] initial stages in yeast and the outer metabolic chamber are considered and fresh evidence with respect to the operation of hexokinase is advanced. Rothstein points out that as phosphate is actively taken up and as the inner surface of the outer chamber is not permeable to the free inorganic ions, it may be concluded that it is taken up in esterified form, and as the only reaction in the fermentation of glucose capable of esterifying orthophosphate is the 3-phosphoglyceraldehyde dehydrogenase reaction, the stages of glycolysis in the outer region proceed to this stage. In addition this author is in favour of the suggestion that the whole of the glycolytic stages occur there. Although the initial loss of energy-rich phosphate bonds is replenished by the dehydrogenase reaction, the transference in the change of enolphosphopyruvate to pyruvate would almost completely exhaust the available free energy of glycolysis and none would appear immediately available to the whole cell. Also, Rothstein considers that pyruvic acid enters freely as such into the inner cell region, whereas the permeability studies already reviewed show it to enter at a very slow rate.

From the above it will be seen that active transport of glucose as such into the yeast cell does not appear to occur, but rather as a phosphorylation which may be combined with some further and so far undetermined stages of the glycolytic process.

The active transport of potassium

In this connexion one can speak not only of an uptake but of transport of potassium ions. Resting yeast in suspension is only very slowly permeable to K. In 5 hours at room temperature, only about 1–2 mmoles per kg. enter from 0·1M-KCl as can readily be shown by labelling the external potassium with ^{42}K. If the suspension is shaken with oxygen, labelled K^+ enters rather fast, so that this exchange appears to be an active one and is inhibited by cyanide (Fig. 1).

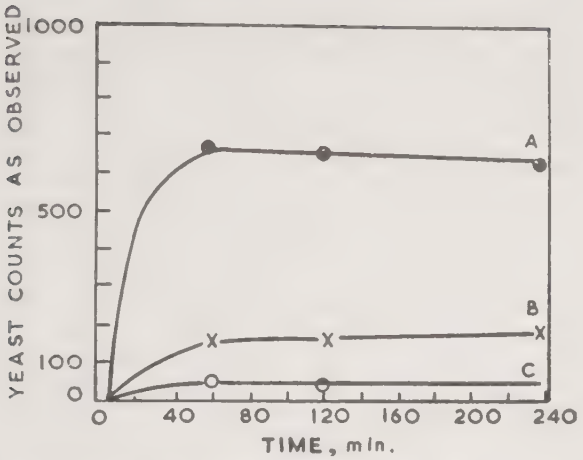

FIG. 1. *Relative rates of entrance of K into untreated yeast* (1 *in* 20 *suspensions*) *in labelled* 0·1 M-*KCl with and without inhibitors*

Curve A without inhibitors; curve B with azide (0·002M) in the suspending fluid; curve C with cyanide. The points give the counts for the yeast, after subtraction of the 5-min. values (Conway *et al.*[16]; courtesy of the *Biochem. J.*)

Even anaerobically, K^+ ions can be taken up rapidly and in large amounts by yeast when it begins to ferment. If the experimental suspension be such that 1 part by weight of washed centrifuged baker's yeast is suspended in 0·6 parts of 5% glucose containing varying amounts of KCl, the amounts of potassium taken up are shown in Table II. If the fermentation is conducted at pH 6·0 to 7·0, using a buffer solution, about twice the amount is taken up. At such pH levels and with large amounts of suspending fluid, the maximum uptake occurs at an external concentration of about 2mM and after about 30 minutes the uptake ceases.

Table II

K^+ and H^+ ion exchange in fermentation

Concn. of K^+ ions in centrifuged fluid		pH of centrifuged fluid after 20 min.	Increase of concn. of free H^+ ions allowing for activity correction (mM./l.)	Decrease of concn. of K^+ ions (mM./l.)
At zero time (mg./100 ml.)	After 20 min. (mg./100 ml.)			
17	9	2·62	3	2
80	39	2·18	8	10
147	88	2·00	11·5	15
330	234	1·77	20	25
1760	1650	1·78	21	28

The fermenting mixture contained 1 part of yeast to 0·5 part of KCl solution plus 0·1 part of 30% glucose and no added buffering (data from Conway & O'Malley[6])

The nature of the potassium uptake

Where the suspension contains 1 part of yeast with 0·6 part of medium, it will be seen from Table II that approximately the same amount of free H^+ ions is excreted as K^+ ions taken up. Without any KCl the acid secretion is shown in Fig. 2 and for yeast oxygenated for 30 hours, in Fig. 3. In the latter case, samples may be obtained which show very negligible increases in organic acid yet the

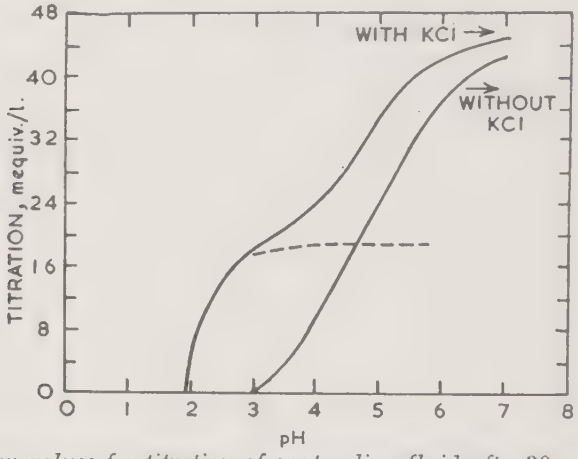

FIG. 2 *Mean values for titration of suspending fluid after* 30 *min. fermentation.* Baker's yeast at room temperature in the proportion 1 kg. of washed and centrifuged yeast to 0·6 litre of 5% glucose with and without 0·1 N-KCl (Conway *et al.*[7]; courtesy of *Biochem. J.*)

free H^+ ion concentration arising from K^+ and H^+ exchange reaches a maximum (see Table III). From such results it must be concluded that K^+ ion is taken up in exchange for H^+ ion but these H^+ ions do not derive from organic acids.

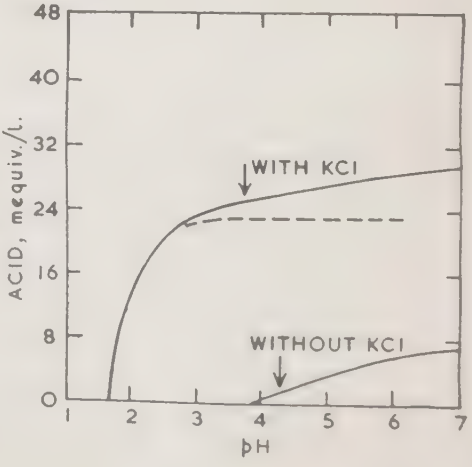

FIG. 3 *As Fig.* 2, *with prior oxygenation of the yeast for about* 30 *h.* (Conway *et al.*[7]; courtesy of *Biochem. J.*)

Table III

Behaviour of succinic acid during fermentation after prolonged oxygenation

Time of fermentation (min.)	pH of suspension	Suspending fluid		Yeast cells	
		Succinic acid (mequiv./l.)	Total ether-extractable acids (mequiv./l.)	Succinic acid (mequiv./l.)	Total ether-extractable (mequiv./l.)
0	—	0·0	1·0	1·4	13·3
24	1·53	0·0	2·9	4·6	15·2

The succinic acid concentrations are expressed with reference to one litre of the suspending fluid or outer medium. This experiment shows the practical disappearance of succinic acid from the suspending fluid (0·6 parts of 5% glucose with M/5 KCl to 1 part of yeast) after prolonged oxygenation, and yet the maximum free acid is reached.

The effect of redox dyes on the K^+ uptake and H^+ ion excretion

A series of redox dyes in very small concentration (10^{-4}M) were investigated for their action on the K^+ uptake and H^+ excretion (Conway & Kernan[8]). These are shown in Table IV and it will be seen that under the conditions used (5% glucose buffered with 1/20 volume of 0·05M-potassium hydrogen succinate) the production of neither CO_2 nor of ethanol is affected, but the uptake of K^+ and excretion of H^+ ions are greatly affected and in relation to the external redox potential (see Figs. 4 and 5).

Table IV

Transport of ions across yeast cell membrane
(Production of CO_2 and ethanol during anaerobic fermentation of yeast with redox dyes in the suspending fluid)

Results expressed as % of the values obtained without the redox dye.

Dye	$E_{\bullet}^{1}$ of dye at pH 4·5 (mv)	CO_2 production (% of control)	Ethanol production (% of control)
Phenol-indo-2:6-dibromophenol	+396	101	103
Phenol-indo-2:6-dichlorophenol	+396	101	101
o-Chlorophenol-indophenol	+388	101	99·5
o-Chlorophenol-indo-2:6-dichlorophenol	+385	100	96
o-Cresol-indo-2:6-dichlorophenol	+365	100·5	100
1-Naphthol-2-sodium sulphonate-indophenol	+273	98	100
Nile blue	+ 19	101	102
Janus green	− 100	101	100
Benzyl-viologen	− 109	100	100
Safranine T	− 139	104	96
Neutral red	− 170	99·5	100

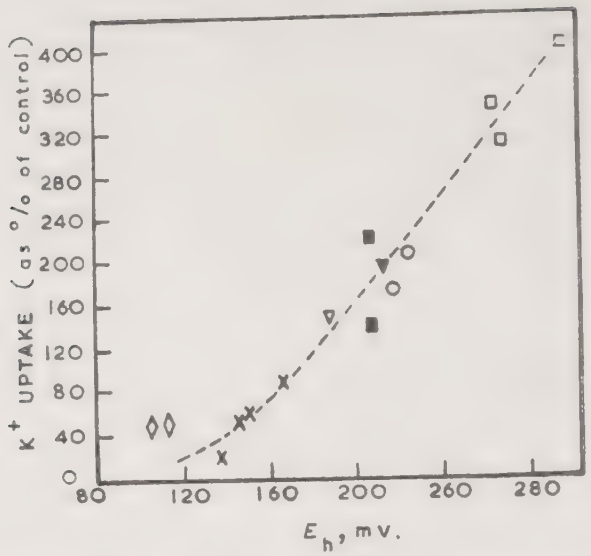

FIG. 4 *Effect of redox dyes on K^+ uptake during fermentation of yeast buffered by 0·04M-potassium hydrogen succinate*
(Conway[9]; courtesy of *Int. Rev. Cytology*)

The dyes used were:

- ◇ Nile blue
- × Safranine T
- ● Neutral red
- Δ Benzyl-viologen
- ∇ phenol-indo-2:6-dichloro-phenol
- \+ Janus green
- ▲ 1-naphthol-2-sodium sulphonate-indophenol
- ○ *o*-cresol-indo-2:6-dichlorophenol
- ▼ phenol-indo-2:6-dibromophenol
- ■ *o*-chloro-indo-2:6-dichlorophenol
- □ *o*-chlorophenol-indophenol

Concentration of dyes 10^{-4} M

How the external redox potential changes during anaerobic fermentation is shown in Fig. 6. In this figure, the first steep fall in the diagram is due to the removal of oxygen after which the potential (E_h) reaches a steady state of about −45 mv. When fermentation occurs (after release of glucose into the mixture), the potential rises rapidly after the initial fall to reach (at pH 4·1) the level of +180 mv in about 1 hour. The incorporation of small concentrations of redox dyes changes markedly the potential at this point; thus *o*-chlorophenol-indophenol increases the level to about + 320 mv and Nile blue decreases it to about 60 mv.

Inorganic redox systems (Ce, Cr, Ti and Sn salts) have a similar but lesser effect on the uptake of K^+ and release of H^+ than that of the redox dyes (Conway[9–11]).

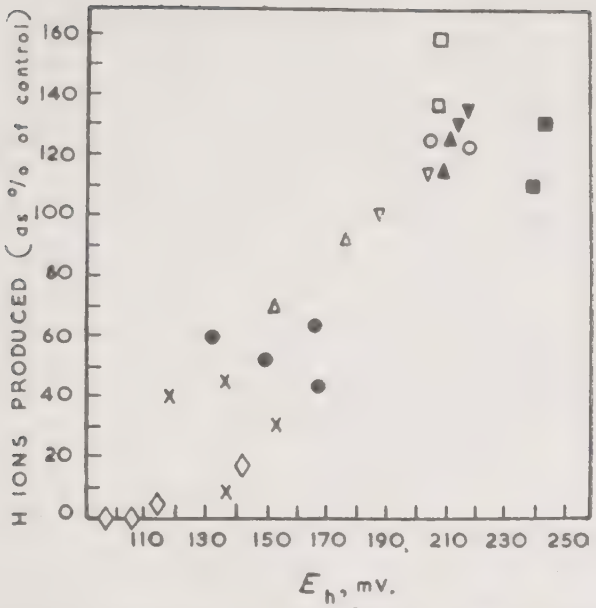

FIG. 5 *Effect of redox dyes on H^+ secretion by yeast fermentating in* 0·04M-*potassium hydrogen succinate at pH* 4·5

Glucose added after expulsion of oxygen. Conditions and symbols as in Fig. 4

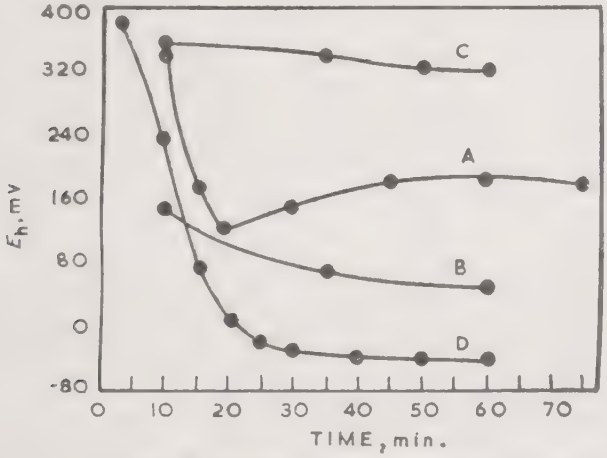

FIG. 6 *Changes of redox potential of yeast*

A fermenting B fermenting in presence of Nile blue C fermenting in presence of *o*-chlorophenol-indophenol D resting Suspending fluid 0·04 M-potassium hydrogen succinate at pH 4·5. A, B and C also contain 5% of glucose; concentration of dyes 10^{-4} M

The redox pump theory for the active transport of K^+ in yeast.

As organic acids of the intermediate metabolic kind do not supply the H^+ ions for the K^+ and H^+ exchange and as redox dyes have a

profound effect in relation to the level of redox potential, it appears probable that the active carrier is a redox system and the fact that the external redox potential is approximately + 180 mv would agree with the view that it is a metal catalyst of the cytochrome type. This is further supported by the effect of pH on the redox potential. For a metal system of the type mentioned, a change of pH should have no effect, whereas if one or two hydrogen atoms were being transferred each unit change of pH should change the potential by 30 or 60 mv.

It has been experimentally found (Conway & Kernan[12]) that the effect within the region pH 4–6 is in fact very low (only 8 mv per unit of pH), the small effect found being attributable to some degree of participation by other systems.

The essential action of the carrier may be represented as follows. Metabolic H atoms, coming say through the flavin system, are received by this metal catalyst and changed to free H^+ ions and electrons retained by the carrier. This then forms a complex with the K^+ ions, which by thermal agitation moves to the other side of the membrane and the electrons of the metal catalyst are transferred to an acceptor, K^+ being set free, and the cycle is completed. In Fig. 7 is given one mode of representing the cycle. This is given for anaerobic conditions, the pathway of carbohydrate metabolism being included in the diagram, with some stages omitted for convenience. Under the diagram three systems are given, the first being the final accepter system in the diagram. The second system indicates how the redox potential of this system can be raised by phosphorylation of the reduced form (RH_2) and so removing it. In the third system it is intended to indicate that the phosphorylated form (RH_2P) can in turn be oxidized. If the P in the oxidized form is an energy-rich bond, the redox potential of the third system will be higher than that of the first. The introduction of these systems here shows how phosphorylation may be used to guide electron transfer.

The nature of the energy transfer in this process, or the thermodynamics of the mechanism, have been dealt with in detail elsewhere (Conway[11]) and such thermodynamical treatment of the redox carrier is essentially the theory of the redox pump. It may be noted that a similar view with regard to the active transport of anions in the root hairs of plants was advanced by Lundegårdh (for a review of his work on active transport see ref. 13).

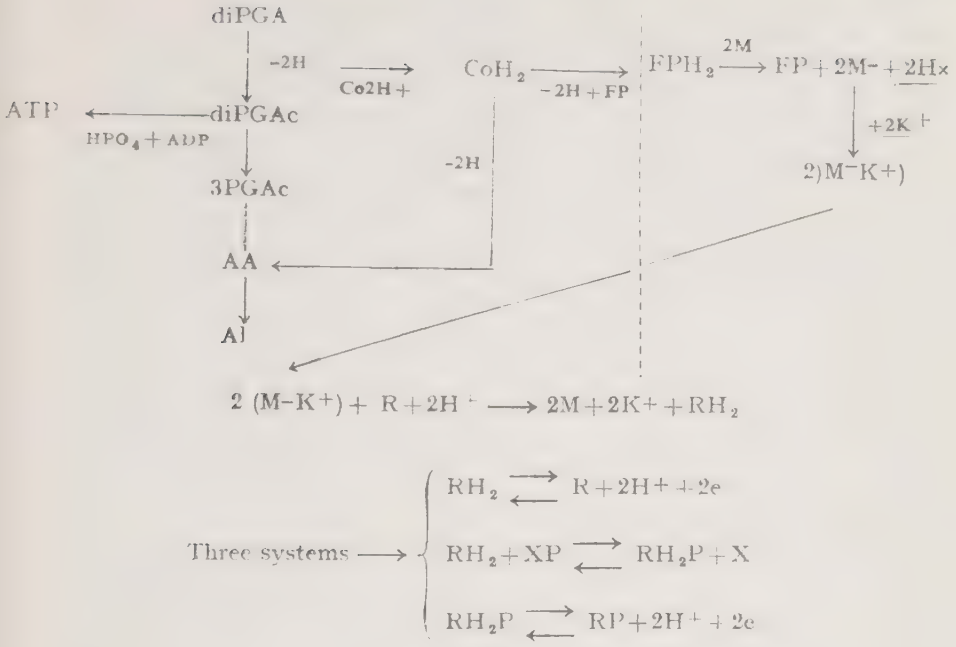

FIGURE 7

ATP = adenosine triphosphate; ADP = adenosine diphoshate;
diPGA = 1:3-diphosphoglyceraldehyde; 3PGAc = 3-phosphoglycerate;
diPGAc = 1:3-diphosphoglycerate; AA = acetaldehyde;
Al = alcohol; FP = flavoprotein;
Co = diphosphopyridine nucleotide (co-enzyme 1)

The K^+ carrier as a general cation carrier

While this carrier is physiologically speaking the K^+ transporter in yeast, it can also carry other cations of the alkali and alkaline earth series but with much lesser affinities. Thus Na^+ must be present in over 25 times the concentration of K^+ to be carried at the same rate, and Mg^{2+} in about 300–800 times the relative concentration. If, however, these other cations are the only ones present (apart from the minute H^+ ion concentration at pH 6 or 7) and in high concentration (they can be accumulated to a surprising extent, magnesium to upwards of 100 mmoles/kg. yeast), but even the presence of 2 mM-K^+ in the external fluid is sufficient to inhibit very largely the uptake of Mg^{2+} by this carrier, with Mg^{2+} present at 200 mM concentration. The relative affinities for various cations have been given by Conway & Duggan.[14]

The active transport of Mg^{2+} by this carrier is of special interest since it requires the presence of oxygen, and is inhibited by cyanide,

whereas the transport of K^+ is largely independent of these factors. This at first might suggest, and rather strongly, that there are two separate carriers, but the ease with which K^+ ions completely inhibit this Mg uptake can scarcely have any other explanation than that they are the one carrier, but that in accordance with the scheme stated the electrons cannot be transferred from the magnesium complex except to the upper cytochrome system and to oxygen. It is also obvious that although its carrier may be made to transport large amounts of magnesium, it is not the normal physiological carrier since it may be assumed that K^+ ions will always be present in sufficient relative amounts to inhibit the transport almost completely. As will be shown below, the physiological uptake of Mg^{2+} operates at a pH level of about 4·5 and is taken in conjunction with phosphate and probably as an undissociated magnesium phosphate.

The sodium pump in yeast

The general cation pump can be made to take up Na^+ in quantity by fermenting in the absence of K^+ but in the presence of 0·2M-sodium citrate. For the suspension of yeast, 1 part of yeast is mixed with 20 parts of 5% glucose and 0·2M-sodium citrate, then 2 hours after the onset of fermentation about 60 mequiv. of Na/kg. yeast has entered the cells, which can be washed a few times and still retain most of the Na. The yeast has lost about as much K^+ as it has gained Na and this process can be repeated, so that after many fermentation cycles practically all the K^+ has been replaced by Na^+ (Conway & Moore[15]).

Consideration is given below to only one cycle with a yeast containing 60 mequiv. of Na and about 70 mequiv. of K per kg. Such a yeast if immersed in water excretes Na^+ along with bicarbonate, acetate and succinate anions (Conway, Ryan & Carton[16]), so that after about 120 min. 40% of the sodium is excreted. If 0·1M-KCl is included, the Na^+ excretion goes two or three times as fast and now largely in exchange for K^+, which is taken up by the K^+ carrier. That the carriers are distinct is shown by the fact that the extrusion of Na^+ is very specific with respect to K^+ as no measurable amount of K^+ is excreted with a Na^+ : K^+ rate of 60 : 70. If the Na carrier had the same affinities for Na and K as the K carrier, then almost 50% of the outward transport of cations would occur as K^+ ions even if only 2 to 3 mequiv. of free K^+ existed in the cytoplasm, but, in fact, no appreciable amount is excreted from a Na-rich yeast containing about 70 mequiv. of K/kg. The difference between the carriers is also shown by the effect of inhibitors. Azide (2mM) in the external fluid at pH about 6·5 inhibits entirely the K^+ uptake but

does not affect the Na^+ excretion in water. In the presence of 0·1M-KCl, it reduces the Na^+ excretion to the level of the excretion in water.

In the presence of Na^+ outside the yeast membrane as well as of 0·1M-KCl, the excretion of Na^+ can be maintained against high gradients. Thus it requires about 500 mM of external Na^+ before the excretion is 50 % inhibited.

The mechanism of the Na^+ transport can be considered as a redox system similar to that of the K^+ uptake, but with special affinity for Na (as compared with K).

Resumé of evidence in support of the redox pump theory

Organic acids as metabolites do not supply the H^+ ions exchanging for K^+ ions nor would phosphate groups appear to be the immediate carriers of K^+, since one would naturally expect in this case that Mg^{2+} ions would have a much greater affinity for the carrier whereas, in fact, it has much less.

Since redox systems, e.g., dyes and inorganic substances, acting anaerobically in very small concentration, do not affect the extent of fermentation but greatly affect the K^+ uptake and H^+ excretions, there is strong evidence for the theory.

The effect of inhibitors may also be mentioned. The general cation carrier can transport K^+ anaerobically and is not then inhibited by cyanide, whereas when it transports Mg, oxygen is required, and cyanide as well as azide inhibits. Dinitrophenol (2 mM) does not inhibit the carrier which is transporting Mg^+ from a 0·2M-magnesium acetate solution, showing that here energy-rich phosphate bonds are not essential to the carrier *per se*, and have only an indirect effect in other conditions. The effect of 2mM-azide added after 30 minutes to the suspending fluid (1 part of yeast to 1 part of 5% glucose in 0·1M-KCl) is a curious one. Not only is any further uptake of K^+ and release of H^+ inhibited, but there is a rapid entrance of H^+ in exchange for K^+, so that the external fluid approaches a neutral solution of potassium succinate and potassium chloride.

The effect of metal inhibitors on K^+ uptake into a Na^+-rich yeast (Conway *et al.* [16]) is also of interest and depends on the two factors: (i) The larger the metal ion, the greater the inhibition; (ii) The degree of inhibition depends on there being 18 electrons in the second outermost orbit and not more than two electrons in the outermost orbit. Uranium, a powerful inhibitor, would appear an exception, but the uranyl and not the uranium ion was used here.

The following is also a fact of much interest. When K^+ is actively taken up in exchange for H^+ ions, while the latter do not come directly from the H^+ ions in the cytoplasm, none the less, according to the redox theory, as each equivalent of H^+ ions is excreted an equivalent of H^+ ions should disappear from the cell. This in fact, has been demonstrated[7] for short-period fermentation as when yeast is suspended in an equal weight of suspending fluid containing 5% glucose and 0·1M-KCl. Under these conditions practically the same amount of H^+ ions is withdrawn from the cells as is excreted into the external fluid. The pH of the yeast cells rises markedly from 5·8 to between 6 and 7.

Where larger volumes of suspending fluid are used, succinic acid forms steadily within the cell and counteracts the H^+ ions withdrawn, the succinate ions so formed being then excreted externally (this agrees with Kleinzeller's experiments[17]).

The succinate excretion from cells in short-period fermentation comes almost altogether from succinate already therein (Brady[18]).

Is there a general anion carrier ?

There are grounds for assuming the existence of an active anion carrier which removes anions from the cell. Thus the lipid membrane is practically impermeable to succinic acid and to succinate, yet succinate is freely excreted from the cells during fermentation. It is conceivable that such succinate ions are forced across the membrane by a steep potential gradient. Succinate ions are also excreted by the cells during the excretion of Na^+ from Na^+rich yeast, and they are accompanied by acetate and bicarbonate ions (but curiously enough by scarcely a trace of phosphate ions). If an active anion carrier existed it would appear largely non-specific.

The physiological magnesium carrier

It has been considered above that the physiological K^+ carrier, although it can act as a carrier of Mg^{2+} ions when these only are present, does not normally do so owing to the several hundred times greater affinity of the carrier for K^+ than for Mg^{2+}. Also the K^+ carrier will only transport Mg^{2+} in quantity when the pH is above 6·0. At a pH of about 4·5, however, and in the presence of phosphate, magnesium is actively taken up with phosphate. This has been studied by Schmidt *et al.*[19] and by Rothstein[3] and confirmed by Conway & Beary.[20] The uptake of Mg^{2+} is in fact stimulated by K^+ ions.

The active transport of phosphate

The yeast cell membrane shows a high degree of impermeability to phosphate ions as such.[21, 22] This may be readily demonstrated by using labelled phosphate (^{32}P) in the external fluid. When fermentation occurs, PO_4^{3+} is actively taken up, but to a much smaller extent than the K uptake (Rothstein & Enns[23]; Conway & Moore[15]).

As labelled phosphate taken up in this way does not appear to mix directly or immediately with inorganic phosphate within the cell (Rothstein[3]), the transport may be regarded as an uptake by phosphorylation and therefore would occur in the triosephosphate dehydrogenase stage.

The uptake of phosphate with magnesium, has been mentioned above.

The uptake of NH_4^+ by yeast

Normally, resting yeast is practically impermeable to NH_4^+ ions introduced outside the cell as ammonium chloride, but if 5% CO_2+ 95% O_2 be bubbled through the suspension these ions enter very appreciably and with increasing rate as the CO_2 tension is increased.[24]

A similar occurrence may be observed in tissue cells of higher organisms, e.g., the frog's sartorius muscle, and may be interpreted as a passive entrance due to carbamic acid formation. During fermentation, NH_4^+ can be taken up by two routes, either in this way or by the K^+ carrier, the affinity of NH_4^+ related to K^+ being as 1 : 10. If fermentation be carried out with 0·1M-NH_4Cl outside the cell without other salt added to the suspending fluid containing 5% glucose, NH_4^+ can be steadily accumulated in the cell, exchanging for K^+. Finally, it is possible to obtain yeast cells in which all the K^+ ions are replaced by NH_4^+. The fate of the NH_4^+ ions after uptake by way of synthetic reactions no doubt involves various metabolic reactions and energy input (see Holzer, p. 55 above).

As is well known, the nitrogen required for growth is not confined to the ammonium system *per se*, but can be supplied by various amino-acids.

It is of some interest to note here that with baker's yeast the K^+ carrier can take up the diamino-acids lysine, arginine, ornithine and histidine at very appreciable rates (Conway & Duggan[14]).

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FOURTH SESSION

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Chairman: Dr. A. J. AMOS

YEAST GENETICS IN INDUSTRY

By R. B. GILLILAND

(*Arthur Guinness Son & Co. [Dublin], Ltd.*)

Introduction

In the year 1935 Prof. Winge published his first paper on yeast genetics,[1] and in 1937 with Laustsen described how the asci of sporing yeasts could be dissected and the single spores isolated and cultivated,[2] while in 1938 the same authors described how they were able to produce interspecific yeast hybrids under continuous microscopical observation.[3] This was the starting point for the systematic investigation of yeast genetics as the main facts of inheritance were then established.

These were

(1) that in the Saccharomyces investigated the normal cell is diploid,

(2) that in vegetative reproduction the cell produces a diploid bud which grows into an exact replica of the mother cell,

(3) that in sexual reproduction 1–4 small spherical spores are formed within the parent cell or ascus,

(4) that although these spores are haploid they can reproduce by budding and can be maintained over long periods as haploid cultures, and

(5) that single spores placed in contact sometimes mate to give a diploid hybrid yeast.

In what follows is examined how yeast genetics has developed during the past 20 years. The technique and basic theory on which yeast genetics depend must first be considered very briefly; and then some typical investigations will be discussed and an evaluation attempted of what industry has gained, or may hope to gain, from this study.

Progress in the use of this new technique was at first slow according to modern standards. Since 1935 there has been a succession of papers from Winge and his associates, first Laustsen and then Catherine Roberts. Lindegren and Mrs. Lindegren were next in the field, and since 1943 a steady stream of papers has come from them and their co-workers. They early made the interesting and important discovery that some yeasts are heterozygous for a strong mating type allele. This means that in a four-spored ascus two spores are of one mating type (a) and two of the other (α) and that mating takes place preferentially between spores of opposite mating types. Spiegelman began publishing in 1945 and has produced many papers mainly on yeast adaptation and enzyme formation. In 1949 Ephrussi

& Slonimski described their discovery of cytoplasmic inheritance, but it is only in the last five or six years that many papers have come from other sources than these.

This slow development may be partially due to the initial difficulty of the techniques used, although this has been largely overcome by the use of modern types of micromanipulator. It is due, however, to a greater extent to the inherent difficulty and slowness of the work.

Production of hybrid yeasts

The essential points in the production of a yeast hybrid are shown in Fig. 1: (*a*) the induction of sporulation in two yeast cultures; (*b*) the isolation of viable spores from each culture; (*c*) the arranging of suitable conditions for mating; (*d*) the production of a hybrid.

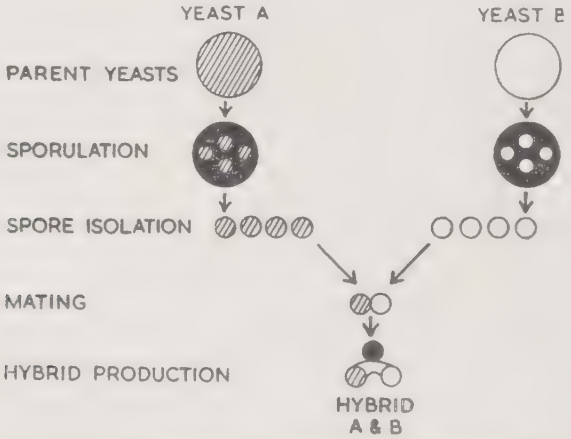


Fig. 1. *Production of hybrids in Saccharomyces*

This simple scheme may break down at any stage. As far as top-fermentation brewing yeasts are concerned, difficulties are encountered in the very first steps. The yeasts will not sporulate, or if they do form spores, these may have a very poor viability when isolated.

Numerous methods have been suggested for inducing yeasts to sporulate or to improve their sporulation, but the most practical methods are the classical moist gypsum block and the sodium acetate–agar medium as described by Fowell[4] and others. A recent suggestion by Wickerham & Burton[5] for dealing with poorly sporing yeasts appears to hold great promise and it is being tried at present. His method is based on the fact that yeast spores are slightly more resistant to heat than are vegetative cells (unlike bacterial spores the difference in resistance may be very small). The technique is to

subject to heat treatment a suspension of the best available sporulating culture to kill the vegetative cells and then to plate it out. Small colonies are selected and, after further purification, these cultures can be suitably tested to see if they are in fact single-spore cultures. By this means spore cultures could be obtained from a yeast which gave only one viable spore in 1000. This would be an impossible case for the direct isolation of spores by micromanipulator.

If we succeed in obtaining viable spores from two yeasts and put them in contact, they may be quite unwilling to mate. In such a case Lindegren's technique may be successful. In his method the isolated spores are allowed to grow into cultures, which are mixed so that hybrids may be formed on incubating the mixture. This mass mating should be followed by Fowell's refinement of isolating single hybrid cells from the mixture and of using them for future work.

Yeast hybrids in industry

The obvious consequence of the knowledge that yeast can be hybridized is that attempts should be made to produce new and better yeasts by breeding programmes similar to those which have had such success in barley, maize and other cultivated plants. Apart from the difficulty of obtaining hybrids from some yeasts, there is also that of determining whether a new yeast is better than the yeasts already available. In one of his very early papers Winge says of this problem: 'This difficulty is particularly pronounced with regard to beer yeast and wine yeast, as no objective method for analyzing the quality of the yeast is available. . . . Undefined demands cannot be answered to order'. In a paper given just a few months ago Lindegren says 'If the brewer can prepare the specification, the geneticist can tell him what characters are hereditary and what kinds of yeast he can produce', but within the knowledge of the author there is no case where an experimentally produced hybrid yeast has been used in commercial brewing of beer.

The yeast geneticist blames this lack of success on the brewer who does not describe in explicit terms what he wants. The author has received from brewers a specification and a programme of investigation has recently been mapped out but, so far, no results have been obtained. Even if success is achieved in producing hybrids of brewery yeasts, it is doubtful if they will be an improvement on the strains which have emerged by the continual selection which has taken place in the brewing process over many years.

The picture is by no means so disappointing in industries other than brewing. In alcohol production and in yeast manufacture an improvement is much more easily measured. Where an improvement in a single criterion (such as rapidity of gas production, or yield of

yeast, or vitamin content) is required, then yeast genetics and a programme of hybrid production may be of value. Winge reported that one of his early hybrids was successfully used for the production of baking yeast, while Lindegren has mentioned the use of hybrid yeasts by Anheuser-Busch, but there is no published account of the planned production of yeast hybrids for commercial purposes and of measurements of the improvements obtained by the use of such yeasts. Such work is, however, in progress and some success has been achieved.

Lindegren[6] has recently listed 13 characters (to which we might add a fourteenth) which are more or less under genetic control. These are shown in Table I. Some of these characters may be rather less than more under genetic control, but, as it stands, it is quite a formidable list. An improvement in some of these characters must surely be an attractive proposition to industries using yeast.

Table I

Genetically controlled characters in Saccharomyces

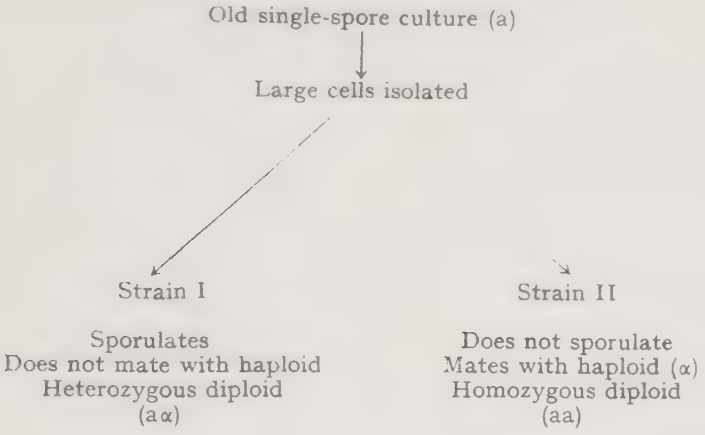
Cell type	Resistance to	Ability to synthesize	Content of
Size	Autolysis	Amino-acids,	Respiratory enzymes
Flocculation	Alcohol concn.	purines, pyrimidines,	
Colour	Salt ,,	B-vitamins	Specific carbohydrases
Growth rate	Sugar ,,		
	Cation ,,		

Polyploid yeasts

Apart from improving yeasts by hybridization, it may also be possible to improve strains by producing polyploid yeasts, i.e., yeasts whose cells contain more than the normal complement of chromosomes. In Saccharomyces the normal yeast is diploid (two sets of chromosomes) and the spore or spore culture is haploid (one set of chromosomes). When a haploid single-spore culture has been kept for some time and sub-cultured at intervals, larger diploid cells may appear and can be isolated (Table II). These cells may be of two different types. They may be heterozygous for mating-type aα, in which case they will readily form spores on a gypsum block, and they will show no mating reaction when mixed with either type a or α haploid cultures. This type of diploid presumably arose from mutation of a mating-type gene in one haploid cell followed by fusion with an unmutated cell. The second type of diploid cell, which may sometimes be isolated from a haploid culture, will not readily

Table II

Occurrence of diploids in a haploid culture



sporulate and when it is mixed with haploid clones it shows a mating-type reaction. It has been shown that cells of this type are homozygous diploids and that when they are mated with haploids they give rise to triploid cells (three sets of chromosomes) as shown in Table III. They may also mate with similarly derived homozygous diploid cells of opposite sex to give tetraploids (four sets of chromosomes). The cells in these triploid and tetraploid cultures are larger than the normal diploid cells (Table IV).[7] They also ferment faster than the diploid cells and give higher yields of yeast under strongly aerobic conditions. The larger cell size might give easier filtration or separation of the yeast from mother liquor and the faster fermentation and growth might be economic of importance. Apparently polyploid yeasts have not been used in industry although they appear to have attractive qualities. Perhaps they have corresponding disadvantages and it may be that they are not stable and readily revert to diploid-type cells.

Table III

Production of triploid and tetraploid yeasts

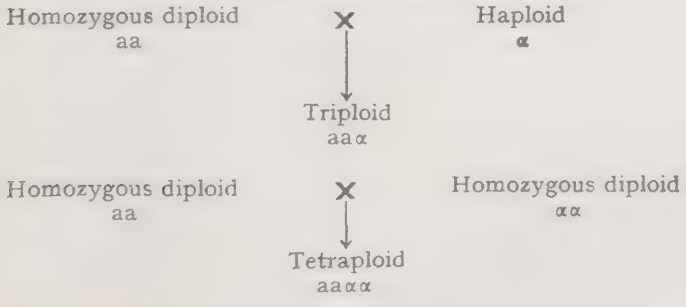


Table IV

Weights and analyses of haploid, diploid and polyploid yeasts
(μg./10^6 cells)

	Haploid	Diploid	Triploid	Tetraploid
Dry weight	14	27	34	73
Metaphosphate ...	0·1	0·16	0·22	0·36
DNA phosphorus ...	0·0023	0·0046	0·0062	0·0094
RNA phosphorus ...	0·055	0·096	0·164	0·172

Yeast genetics as a source of information

Although as yet no hybrid or polyploid yeast may have been used in commercial brewing, this industry and other yeast-using industries have gained in other ways from the study of yeast genetics. The brewing industry is going through the period of finding scientific reasons for empirical knowledge, e.g., why the brewer prefers certain malts; what are the chemical constituents of hops; and what the yeast cell does and how it does it. The reason for this search for knowledge is to provide a solid basework of fact from which improvements may be made. The study of yeast genetics has played its part in this endeavour to discover the reasons and the mechanisms behind the empirical facts of yeast growth and fermentation.

Inheritance in yeasts may be studied by forming a hybrid between two yeasts which differ in some character and then investigating the segregation of this character in spores obtained from asci of the hybrid. The example given in Table V is of the ability to ferment maltose.[8] Two out of four spores in each ascus of the hybrid have the dominant character and two the recessive. We obtain this simple and exact demonstration of Mendelian inheritance because in yeast the

Table V

Inheritance of maltose fermentation in a hybrid

S. chevalieri (mm) × *S. italicus* (MM)

↓

Hybrid (Mm)

↓

Segregation in 32 four-spored asci.	Maltose fermenters M	:	Maltose non-fermenters m
32	2	:	2

haploid spore can give rise to an independent, freely-growing culture whose characters can be determined. There is then no concealment of recessive characters so that it is not necessary to produce back-crosses in the next generation and treat the results statistically, as is necessary with plants or animals which have a free existence only in the diploid form. In most other organisms a deviation from the 1 : 1 ratio in the inheritance of a character could not be detected unless it was statistically significant over a long series. In yeasts, a single deviation from the 1 : 1 ratio can readily be detected and can then be further investigated. Yeast genetics is therefore of interest both from the light it throws on the mechanism of the characters investigated and also for the information it gives on the way inheritance takes place.

In yeast asci occasional deviations from the 1 : 1 ratio sometimes occur in a character which is known to be governed by a single gene. Some of these abnormal segregations have been shown to be due to gene mutation, some to polyploidy, some were caused by extra mitoses within the ascus giving rise to diploid spores, while others have been explained by the occurrence of gene conversion. In gene conversion it is assumed that the dominant allele becomes recessive, or vice versa, by interaction with the complementary allele during meiosis. If this could be proved to be a normal phenomenon in yeasts it might have an important bearing on genetics in general. It is, however, strikingly difficult to prove or disprove the case for gene conversion and, whether it takes place or not, is still hotly argued between Lindegren and Winge, but further work with yeasts may decide the issue.

It would require too much space to detail all the results obtained by means of genetic analysis and only a few will be mentioned which may be of interest to brewers and yeast industries. These are yeast flocculation, fermentation of carbohydrates such as maltose, maltotriose, dextrin and starch, and the isolation of enzymes by means of genetic segregation.

Yeast flocculation

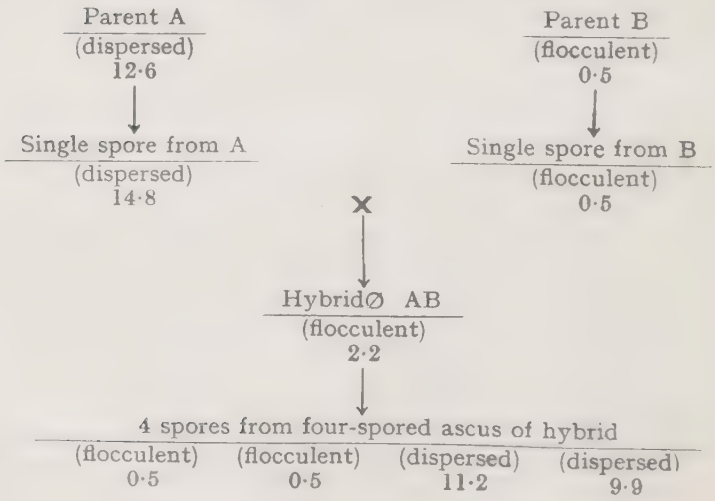
Yeast flocculation was, and still is, a perplexing problem. On numerous occasions trouble has arisen in a brewery through a brewing yeast becoming more and more flocculent in successive fermentations. This is generally accompanied by slower fermentations, poor attenuation and sometimes by failure of the yeast to give a good crop on skimming. There have been many arguments as to whether such a change in brewing yeast was due to the influence of the yeast growth medium or to a change in the yeast cells or to

invasion by another yeast. By crossing a flocculent with a non-flocculent yeast and analyzing four-spored asci of the hybrid, it has been shown that one type of flocculence, at least, is genetically controlled and is governed by a single gene. An example of this is given in Table VI.[9] This has been independently confirmed with a number of different yeasts. In one yeast, however, flocculence was governed by three genes, any one of which could confer the flocculent character to the yeast containing it.[9a] A study of the mutation rate of the gene for flocculence has shown that, while mutation from the dominant (flocculent) to the recessive (non-flocculent) occurred with high frequency, the mutation (non-flocculent→flocculent) occurred extremely rarely.[9a] In a very recent paper by Jeffery[10] these comparative rates are confirmed, i.e., mutation generally occurs in the direction flocculent→non-flocculent. This, however, is the reverse of the direction which normally causes trouble in top-fermentation breweries. It may be therefore concluded that mutation in itself cannot be the cause of this change of yeast type which causes breweries so much concern.

In connexion with flocculation, an extraordinary phenomenon has recently been described by Wickerham.[11] A newly isolated species of *Hansenula* (which he has named *Hansenula Wingei*) was induced to spore, and single spore cultures were isolated, which showed the mating-type reaction. When cultures of spores of the same sex were mixed, a creamy mass of cells which dispersed completely in water

Table VI

Inheritance of flocculence
(figures are g. of yeast/l. in suspension)



was obtained, but when cultures of spores of opposite sex were mixed, in a few seconds the cells set to a solid mass which would not disperse in water. The reason for this mutual preciptation is not yet known but its elucidation may well throw light on the general problem of flocculation.

Sugar fermentation

A large part of the published work on yeast genetics is concerned with sugar-fermenting ability. This is because yeast species show easily measured and sharply defined differences in their ability to ferment various sugars, so that this is an easy character to study. It has been shown that fermentation of a sugar may be governed by a single gene pair: this gives two fermenters and two non-fermenters in each four-spored ascus. Fermentation may also be controlled by two or three gene pairs (multiple alleles). An example of this is the inheritance of raffinose fermentation in the same hybrid which is governed by three genes (Table VII).[8] In this case when the hybrid is analysed a preponderance of asci containing either four fermenters, or three fermenters and one non-fermenter is found.

Another distribution of fermentative ability occurs when two or three complementary genes are required to cause fermentation. When this happens, the four-spored asci of a hybrid with a yeast recessive for these genes gives 0, 1 or 2 fermenters. An example of this is the inheritance of the ability to ferment galactose which in some cases is governed by two complementary genes.[11a] In each of the last two cases the ratio of the number of different types of asci follows a recognized pattern.

Table VII

Inheritance of raffinose fermentation in a hybrid

S. chevalieri $(R_1R_1 \quad R_2R_2 \quad R_3R_3)$ × *S. italicus* $(r_1r_1 \quad r_2r_2 \quad r_3r_3)$

↓

(Hybrid) $(R_1r_1 \quad R_2r_2 \quad R_3r_3)$

↓

Segregation in 20 four-spored asci	Raffinose fermenters	Raffinose non-fermenters
12	4	0
6	3	1
2	2	2

Recently there has been interest in the fermentation of maltotriose and higher carbohydrates. It has been found that maltotriose (which constitutes 15–20 per cent of the fermentable carbohydrates of brewers wort) is fermented by some strains of *Saccharomyces cerevisiae* and not by others. By crossing a fermenter with a non-fermenter it was shown that maltotriose fermentation is governed by a single gene.[12] Each four-spored ascus of the hybrid gave two fermenters and two non-fermenters except in one case which may be due to mutation (Table VIII). It has been suggested that non-maltotriose fermenters should be removed from the species *S. cerevisiae* and given species rank with a new name because these yeasts could not function as normal brewing yeasts, and because the character is genetically governed, is stable and easily detected.

Table VIII

Inheritance of maltotriose fermentation

S. carlsbergensis (maltotriose +) × *S. cerevisiae* (maltotriose —)

↓

(Hybrid) (maltotriose +)

↓

Segregation in 11 four-spored asci	Maltotriose +	:	Maltotriose —
10	2	:	2
1	1	:	3

Dextrin and starch fermentation

The yeast *Saccharomyces diastaticus* ferments starch and dextrin. Hybrids were made between this yeast and yeasts which did not ferment these carbohydrates and segregation in asci of the hybrids was investigated.[13, 14] Using dextrin as a substrate, the simple 2 : 2 ratio was readily demonstrated in some of the hybrids. In other hybrids 2 : 2 ratio was eventually found, although it was at first masked by the presence of some slow dextrin fermenters. This slow dextrin fermentation was strongly associated with slow maltose fermentation (see paper by Hopkins, p. 31 above).

Recently Lindegren reported[15] independent starch and dextrin fermentation in isolates from a hybrid, one of whose parents was *S. diastaticus*. One gene governed the production of a dextrinase which was able to ferment maltose slowly and to split glycogen, but

it could not attack either starch or amylopectin. A second gene conferred the ability to ferment starch or amylopectin but not dextrin, glycogen or maltose. These two genes segregated independently. The gene governing dextrin fermentation segregated completely regularly (2 : 2), but that for starch fermentation very irregularly. There was an over-all deficiency of fermenters (20 positive to 32 negative) and yet three fermenters to one non-fermenter were found in some asci. This irregular segregation cannot yet be explained. During this work some cultures with most interesting fermentation reactions were produced. For example, one culture fermented dextrin, maltose, and glycogen but neither starch nor sucrose; this culture did not contain the normal maltase gene but it was able to ferment maltose slowly, presumably by the action of dextrinase. Another culture could ferment starch and amylopectin but could not ferment dextrin, glycogen, maltose or sucrose. These yeasts might be useful for the removal of either starch or dextrin from a mixture of the two.

Yeast genetics for the isolation of enzymes

A yeast may contain two enzymes X and Y which are almost inseparable by physical means. If this yeast is crossed with another yeast which is deficient in X and Y, and independent segregation takes place in asci of the hybrid, then cultures can be isolated which contain the enzyme X completely uncontaminated with Y and vice versa. This is a most valuable tool which has as yet been little used in enzyme chemistry, and it may also have some future industrial use for the production of specific substances by selective fermentation.

Conclusion

No mention has been made of the large body of work on adaptation and mutation of yeasts, on cytoplasmic inheritance (see paper by Slonimski; p. 7 above), or of the use of linear asci which give more genetic information than random tetrads. Each of these studies has an important bearing on genetics in general, but even in this restricted survey it has been shown that yeast genetics has produced results of interest to industry even if it has not yet produced a yeast which gives a better beer.

Acknowledgment

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Discussion

Professor R. H. Hopkins: I can confirm that in suitable cases the dissection of ascospores and their subsequent propagation affords an ideal separation of enzymes, more perfect than crystallization, electrophoresis, etc. I have been fortunate enough to secure from Mr. Gilliland cultures of 'single spore yeasts' so separated, and preparations and extracts from these cultures were entirely free from one another's distinctive enzymes.

The recently published results of Lindegren contain some surprising features, but much depended on, among other things, the exact nature of 'dextrin' used by that worker. Would the lecturer state whether the dextrin used in his own experiments contained short chain fission products, e.g., maltotriose, etc., which would move on a paper chromatogram, and whether his dextrin stained with iodine?

Mr. Gilliland: I have no idea of the nature of the 'dextrin' used by Lindegren and very little of the nature of the 'dextrin' I used. I was only concerned to get a substrate which gave a good differentiation between 'dextrin fermenters' and 'dextrin non-fermenters' and a commercial brand of 'pure precipitated dextrin was adequate. It contained no maltotriose or any other compound which gave a spot which moved on a chromatogram and it stained purple with iodine.

Mr. R. R. Fowell: Could Mr. Gilliland specify the nature of the starch used in his demonstration that *S. diastaticus* is capable of fermenting starch? Is it not possible that the starch used (soluble starch) was partially broken down to oligosaccharides, and that fermentation of these gave rise to the impression that it is starch that can be fermented?

Mr. R. B. Gilliland: The starch used may have contained some oligosaccharides, but from the amount of alcohol formed it was established that the starch molecule itself was undoubtedly attacked.

Prof. R. H. Hopkins: I can confirm that a pure starch such as waxy maize starch is attacked by *S. diastaticus*.

Dr. R. G. Ault: (1) In view of the importance of flavour to brewers, will Mr. Gilliland be prepared to say whether flavour production of brewing yeast is likely to be a matter of genetical control? (2) Since maltotriose is largely found at the end of the primary fermentation, it presumably is fermented much more slowly than glucose or maltose. Is any part of the maltotriose, however, fermented during the primary fermentation?

Mr. Gilliland: (1) Flavour in beer is probably to some extent under genetic control, but it is very unlikely that it is under such simple genetic control that it is strongly influenced by one or two genes. In such a case it is very difficult to investigate the inheritance of the character and it is also very difficult to improve such a character by breeding. (2) Maltotriose is fermented slowly during the primary fermentation but towards the end of the fermentation only maltotriose and no maltose is left in the beer.

Dr. H. Jørgensen: Was the maltotriose used devoid of α-1:6-linkages?

Mr. Gilliland: The maltotriose used was glucose-1:4-glucose-1:4-glucose. There may have been traces of other compounds but there was not enough of any of them to give a spot on a chromatogram.

SOME GENETICAL ASPECTS OF PANARY FERMENTATION

By R. R. FOWELL

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Introduction

The application of genetical analysis to a complex process such as panary fermentation is rather ambitious, and may be considered premature as fermentation genetics is a comparatively new study. An extensive study of dough fermentation has been carried out in these laboratories however, and, statistically designed experiments, carried out on a large number of haploid and diploid yeasts, have yielded results, which, apart from their biochemical interest, have helped to throw considerable light *inter alia* on the genetical control of fermentation rates.

A great deal of genetical work has been reported on the qualitative aspects of sugar fermentation, chiefly by Winge & Roberts in Denmark, and Lindegren and his co-workers in America. This work can be summarized in the statement that ability to ferment a sugar is dominant to inability and, for certain sugars, a number of genes is involved. For example, it is now known[1] that six different maltase genes (M_1 to M_6) are responsible for maltose fermentation, and that each of these on its own can induce fermentation. The genes differ biochemically; for example, M_1 hydrolyses maltose and sucrose, while M_2 hydrolyses maltose only. (See also ref. 1a.)

The quantitative aspects of fermentation, however, have been largely ignored from a genetical point of view. A distinction has been recognized, for some sugars such as galactose,[2] between the genes for rapid and for slow fermentation, respectively. Gilliland[3] has shown that M_5 governs slow or delayed fermentation in *Saccharomyces diastaticus*; it would be interesting to know the effect of the other maltase genes on the rate of maltose fermentation. None of these genes operates *in vacuo*, and there are undoubtedly several genes, some perhaps controlling side-chain reactions and others nitrogen metabolism (connected with enzyme synthesis), which directly or indirectly govern rates of sugar fermentation.

Experimental

In the study of dough fermentation mentioned above, the fermentometer of Schultz, Atkin & Frey[4] was used to measure the increase in volume of a standard piece of dough, in a closed system, at regular time intervals. All values for CO_2 mentioned below refer to ml. evolved for 0·5g. wet yeast (27% dry matter) at 30°. The

values considered to be of the greatest importance are those for 1-h. and 1–3-h. fermentometer volumes.

The reasons for choosing these particular values are as follows: when yeast is added to flour and made into a dough, fermentation takes place initially at the expense of sugars present in a free state, e.g., sucrose and levosin, which are first rapidly hydrolysed to a mixture of glucose and fructose (traces of which are in the original flour). As the rate of hydrolysis is normally much greater than the subsequent fermentation of these hexose sugars, it is clear that the rate of fermentation is unlikely to be restricted by this preliminary hydrolysis. This is probably true of most, if not all, haploid yeasts for variations in invertase activity have been shown to be without effect on fermentometer volume.

Immediately flour is moistened into dough, the amylase system starts to break down the available starch to maltose. Yeast selectively ferments first the hexose sugars derived from the free sugars of flour and, when these near exhaustion, commences on the maltose. This occurs after about the first hour of panary fermentation.

Broadly speaking, therefore, the 1-h. fermentometer volume corresponds with the period of hexose fermentation in dough; and the 1–3-h. volume with the period of maltose fermentation in dough. Conditions in a complex medium such as dough are very different from those in simple sugar solutions, and an added complication is that a certain amount of yeast growth occurs in the later stages of panary fermentation, especially in the 2–4-h. period. The rate of fermentation in dough, therefore, may be expected to differ from that of a simple sugar solution containing the sugar assumed to be the substrate in dough. A fairly close correlation has been found to exist, however, between the 1-h. fermentometer volume and the amount of CO_2 evolved on the synthetic medium of Atkin, Schultz & Frey (ASF medium)[5] supplemented with glucose. From a genetical point of view, it is interesting to compare (Fig. 1) the frequency distribution of a number of haploid yeasts for 1-h. fermentometer volume dough fermentation with that for glucose/ASF fermentation volume. Both distributions show the same number and types of peaks (three) which indicate the operation of a few common major genes. Thus genetical analysis of early panary fermentation gives a clear indication of the principal genes directly controlling glucose fermentation.

Examination of fermentometer data for haploid and hybrid yeasts has shown, quite clearly, that variations in 1–3-h. fermentometer values occur independently of 1-h. values, i.e., the two stages of panary fermentation are under different genetical control.

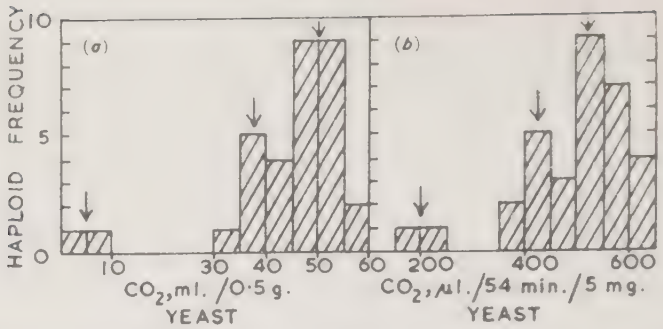

FIG. 1 *Comparison of frequency distributions of haploid yeasts for* (a) *1-h. fermentometer volume (dough fermentation) and* (b) *glucose/ASF fermentometer volume*

Note similarity of peaks indicating operation of a few major genes controlling fermentation.

Measurements of plain maltose fermentation rates (μl./CO_2/60 min./10 mg. of yeast) have revealed that maltose fermentation in dough is considerably influenced by factors largely of an unknown nature. In one yeast, maltose fermentation rates for haploids are closely correlated with 1–3-h. fermentometer volumes, but even more closely with 1-h. fermentometer volumes. The haploids of another yeast show only weak associations between maltose fermentation and dough fermentation properties.

Difference in rates of adaptation to maltose fermentation may account in part for the above results. Examination of frequency distributions for 1–3-h. fermentometer volume reveals considerable variations among haploid and hybrid yeasts. Generally speaking, these distributions tend to be continuous, but they sometimes show the three peaks (Fig. 4) believed to be indicative of the major genes directly controlling the glycolytic cycle. The fact that these peaks are not so prominent probably means that, in the later stages of panary fermentation, other genes play an increasingly important part in controlling the rate of fermentation.

Yeasts used

Genetical analysis of panary fermentation must be ultimately based on the crossing of different yeasts and the testing of the haploid and hybrid yeasts involved. Some attention must be given to the design of the breeding programme now to be discussed. Two parent yeasts, designated for convenience as X and Y, were selected for this work. These yeasts were induced to sporulate by transfer to sodium acetate agar slants,[6] and the resultant spores were isolated by micromanipulation. Haploid cultures required for breeding were obtained by transfer of spores to malt wort. Plus (+) and minus(–) haploids were obtained from both yeasts. These were

submitted to exhaustive trials in an effort to determine, by statistical analysis, the distribution of errors between different processes of propagation and testing. Estimates were obtained for all possible sources of variation, namely between tests, day-to-day errors, between fermenters, and so on. In this way, it was possible to separate environmental from genetical sources of variation and so to apply suitable corrections. Corrections also had to be applied for the different batches of flour used in successive tests. Similar methods were later applied to the hybrids obtained from the haploids.

A suitable breeding scheme to facilitate subsequent statistical analysis was provided by the formation of a 6×6 block of hybrids obtained by crossing 6 haploids of one yeast with 6 haploids of the other. All hybrids were obtained by Lindegren's mass-mating technique followed by isolation of single hybrid cells.[7] Vertical and horizontal columns of hybrids were tested alternately, i.e., in 12 batches, so that every hybrid was eventually tested twice and this scheme enabled all necessary corrections to be applied for inter-trial and other errors.

Two 6×6 blocks of hybrids were formed as follows:

i. 6 minus (–) haploids of X crossed with 6 plus (+) haploids of Y.

ii. 6 plus (+) haploids of X crossed with 6 minus (–) haploids of Y.

For convenience, these blocks will be referred to as the X – Y + and X + Y – blocks respectively.

Genetical analysis of 1-h. fermentometer volume

(1) *X – Y + block.*—The X haploids formed a genic group quite distinct from the Y haploids (Fig. 2). Thus 1-h. fermentometer volumes (as previously defined) showed a range of 35·5 – 47·0 ml. for X haploids and 49·5 – 54·0 for Y haploids (standard error 0·6). No data are available for one of the X haploids as it could not be propagated on molasses (the medium used for propagation of baker's yeasts both on factory and laboratory scale), probably owing to some biochemical deficiency such as lack of a key enzyme for assimilation.

One-hour volume measurements on hybrids showed that about 20 out of 36 were superior to even the best of the haploids, indicating genic interaction between haploids.

The hybrids (Fig. 2) showed a characteristic distribution, values for 1-h. volume falling into three distinct groups as previously noted for haploids; again, a broken distribution of this kind denotes the operation of a few major genes. The middle group of values fell within a very short range (54 – 56 ml.) indicating that one particular genotype was predominant. The highest group was of a more spreading nature, and was probably accounted for in terms of a few genes

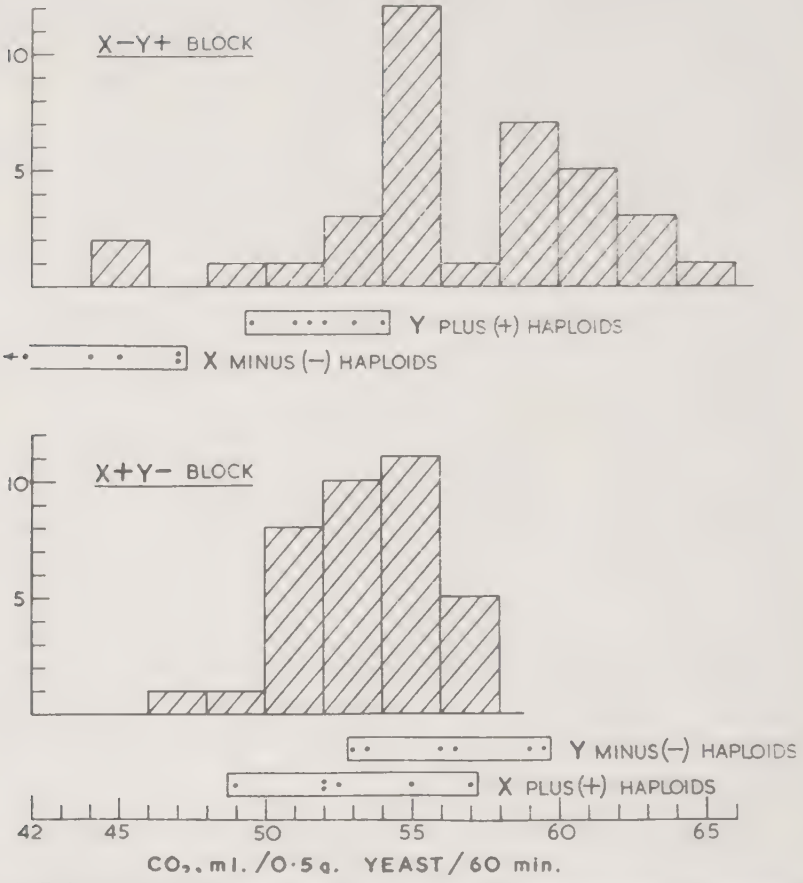

FIG. 2 *Relationship between* 1-*h. fermentometer volume values for hybrids and haploids*

The discontinuous nature of the frequency distribution in the X − Y + block of hybrids indicates the operation of a relatively small number of major genes.

interacting to give different genotypes with slightly different phenotypic, i.e., expression, effects.

The results obtained can be conveniently explained in terms of four major genes, denoted as A, B, C and D, operating with dominance (Fig. 3). The different groups of values are assumed to be due to the presence of one, two and three genes respectively, e.g., genotypes such as AB, BC, and AC account for fermentometer volumes in the 51–57-ml. range. It is also assumed that two of the X haploids possessed a novel gene, D, which in association with AB, gives the best genic combination, ABD; this assumption is based on the finding that the two haploids concerned gave the best series of hybrids.

The segregation of the X and Y haploids into two distinct groups permits the postulation that all X haploids had one gene and all the Y haploids two genes (except 30a, which gave the poorest set of

F.V. 35–50 : ONE MAJOR GENE
51–57 : TWO " GENES
58–65 : THREE " "
ml. CO_2 / 0·5 g. yeast

MINUS (–) X HAPLOIDS

PLUS (+) Y HAPLOIDS		H10 D	9H1 A	9H8 A	19H14 C	28H4 C	H11 D
41C	AC	ACD	AC	AC	AC	AC	ACD
8A	AB	ABD	AB*	AB	ABC	ABC	ABD
20B	AB	ABD	AB	AB	ABC	ABC	ABD
27A	AB	ABD	AB*	AB	ABC	ABC	ABD
18B	AB	ABD	AB	AB	ABC	ABC	ABD
30A	B	BD	AB	AB	BC	BC	BD

ABD GENOTYPE ASSUMED TO GIVE HIGHEST VALUES

FIG. 3 *Genotypes of hybrids prepared by crossing six plus (+) haploids of yeast Y with six minus (—) haploids of yeast X*
For the sake of clarity, all recessive genes are omitted and each capital letter denotes a dominant gene (present singly or in duplicate) contributing towards 1-h. fermentometer volume. The two hybrids marked with an asterisk are characterised by abnormally low fermentometer volumes.

hybrids). The genes for the X haploids must be different to account for the finding that the mean values for the hybrids derived from them fell into three distinct groups; thus two X haploids probably had the same gene, A; two had C; and two had D. Hybrids from the Y haploids had mean values with a narrow range. Thus it is probable that four of these haploids (8a, 18b, 20b, 27a) all had the same genotype, AB. This accounts for genetical interaction with all the X haploids, except two (9h1, 9h8) which must have a gene identical with one of the genes in the AB genotype, e.g., A. The Y haploid, 30a, is allocated the single gene, B, which accounts for the poor types of hybrids obtained from this haploid.

The genetical scheme thus outlined accounts very well for the values recorded for 1-h. fermentometer volumes given by all 36 hybrids except two (indicated by asterisks in Fig. 3): these proved exceptional in giving abnormally low 1-h. and also 1–3-h. volumes; in fact, they were inferior to both their component haploids for these features. This indicates the operation of genes acting in a *negative* direction when suitably combined in a hybrid. Thus if one haploid

possessed a gene, Q, and another haploid a gene, R, both without effect individually on fermentometer volume, then a hybrid prepared from these haploids would acquire the genic combination, QR; it may be supposed that these two genes govern successive steps in a side reaction, and when present together enable this side reaction to take place to the detriment of fermentometer volume.

(2) *X + Y– block.* — In contrast to the first block, both sets of haploids employed here gave significantly better values for 1-h. fermentometer volume (Fig. 2). This block, therefore, was expected to provide hybrids superior to those in the first block; but unexpectedly, the exact reverse was found. The hybrids proved to be all very similar, and formed a compact distribution group corresponding to the middle group in the first block.

Even more remarkable, values recorded for fermentometer volume revealed that, with two doubtful exceptions, not one of the hybrids was superior to the Y haploid from which it was derived. In fact, at least half were significantly inferior to the Y haploid and six were inferior to *both* the component haploids.

These results can only be accounted for in terms of genes acting in a negative direction, and such genes apparently assumed considerably more importance in this block of hybrids than in the first block.

Genetical analysis of 1–3-h. fermentometer volume

(i) *X – Y + block.* — The 1–3-h. measurements for this block showed (Fig. 4) that, in general, the hybrids resembled the haploids from which they were derived. Only about five hybrids were definitely superior to their component haploids. Several hybrids, notably those from an exceptional X haploid (9h1), were inferior to one or other of the component haploids, and some were inferior to both.

The frequency distribution for hybrids showed evidence of three peaks, indicating the operation of major genes. Analysis of the results, however, gave a clear indication of genes operating negatively as well as positively. It has proved possible to draw up a provisional genetical scheme showing interaction between five major genes acting positively and five genes acting negatively. By means of this scheme, it is possible to account for the finding that one haploid (X9h1) gave a mixture of some of the best and worst hybrids.

(2) *X + Y – block.* — Compared with the first block, the Y minus (–) haploids were markedly superior to the Y plus (+) haploids used in that block; and superior hybrids were expected here as in the 1-h. fermentometer block. This expectation was again refuted by the results; the hybrids were comparatively mediocre, forming a relatively compact distribution group corresponding to the middle group in the first block (Fig. 4).

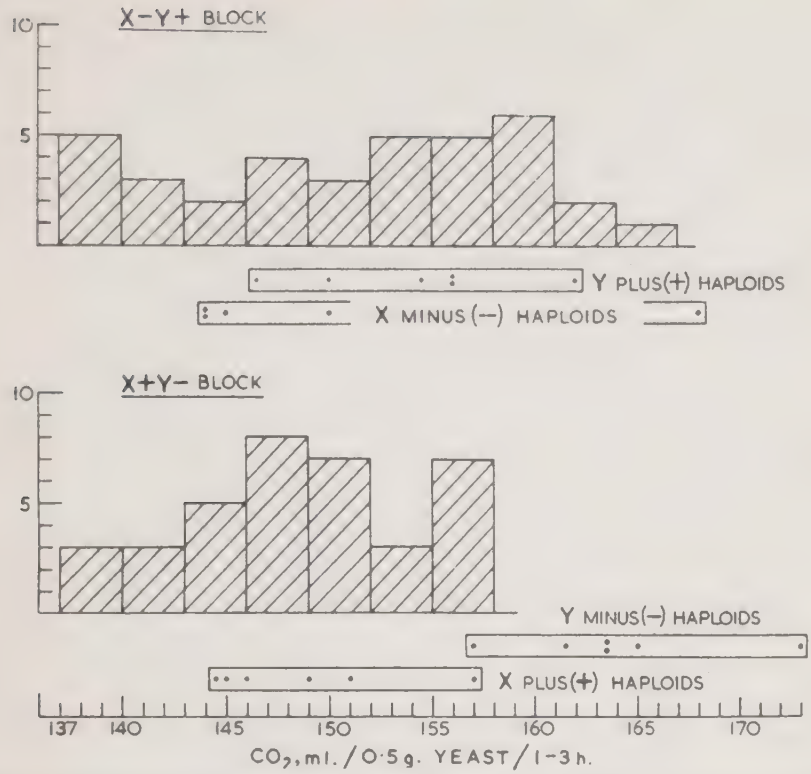

FIG. 4 *Relationship between 1 – 3-h. fermentometer volume values for hybrids and haploids*

The frequency distribution in the X−Y + block of hybrids indicates that, in the later stages of panary fermentation, the action of the major genes controlling fermentation rate is partially obscured by other genes.

Negatively-acting genes dominated the control of 1–3-h. volume to an even greater extent than the control of 1-h. volume in this block. This was evident from the detailed results which showed that only two of the 36 hybrids were even comparable with the Y haploids from which they were derived; all the rest were inferior to their component Y haploids.

Conclusions

The genetical control of gas production in dough is obviously very complex. The results obtained show quite clearly that the rate of CO_2 evolution is governed by several genes, some of which operate in a positive and others in a negative direction. Moreover, in the later stages of panary fermentation negatively-acting genes assume an increasingly important rôle. On biochemical grounds, these results should not be unexpected as the various biochemical pathways in

the yeast cell interlock in a very complicated manner, and quantitative values for features such as CO_2 evolution clearly depend on the balance of numerous reactions controlled by enzymes. Nitrogen metabolism undoubtedly plays a major part in determining enzyme synthesis and adaptation to different substrates, and although relatively few major genes have been postulated to explain the results just discussed, the final figures for fermentometer volume must be governed by numerous other genes with minor effects involved in nitrogen metabolism and perhaps other processes.

The genetical schemes devised to explain the results can only be regarded as provisional; nevertheless, by revealing genetical differences rather than the absolute nature of gene complexes, it is suggested that they can provide much useful information for the purposes of selective breeding. The results show, however, that predictions of individual hybrid performance based on the behaviour of haploids are virtually impossible, and will continue to be so until an extensive scheme of genetical analysis has been carried out. Statistical analysis has, however, served to show that certain haploids tend to give better hybrids than others, and these haploids are, as experience has shown, seldom if ever the best among the haploids available for breeding.

Biochemists naturally tend to concentrate their studies on single strains of organisms, but it is suggested that the breakdown of a diploid organism into a number of haploids offers great opportunities for the study of biochemical equilibria and alternative biochemical pathways in the yeast cell. Some advantage has already been taken of this biochemical phenomenon and, as one example, evidence has been found, from a study of one haploid, for the theory of direct maltose fermentation. This haploid shows a maltose fermentation rate about 25% greater than for glucose: this example should stimulate biochemists to devote more attention to haploids than hitherto.

Acknowledgments

The author wishes to thank his colleague, Mr. Burrows, for his invaluable assistance in arranging the fermentometer tests and organising the statistical analysis of experimental data.

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FIFTH SESSION

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Chairman: DR. L. A. ALLEN

THE UTILIZATION OF FLOUR CARBOHYDRATES IN PANARY FERMENTATION

By R. M. MACKENZIE

(*The Distillers Co., Ltd., Menstrie, Clackmannan*)

The sugars in flour

Since the discovery of levosine in flour by Tanret,[1] there is no doubt that later advances in the understanding of the carbohydrates contained in flour coincided with the advent of paper chromatography.

The free sugars contained in flour were thought to consist of sucrose, glucose and fructose in relatively unimportant concentrations together with levosine, a mixture of oligosaccharides containing glucose and fructose, concerning whose availability as a fermentation substrate conflicting views were held. The main substrate of panary fermentation was considered to be maltose, produced by the action of flour amylase. Colin & Belval[2] estimated the free sugar content of flour and found 0·17% of hexoses, 0·22% of sucrose, and 0·60% of levosine. This work was carried out without the aid of chromatography, and the low figure quoted for the sucrose concentration did not find ready acceptance. The considerably higher values accepted at the time for the sucrose content of flour were probably due mainly to the complex nature of the fructosans classified as levosine, which include easily soluble, non-reducing acid-labile sugars.

Williams & Bevenue[3] examined the sugars of flour by quantitative paper chromatography and reported the presence of fructose, glucose, sucrose and levosine. In the same year, Koch *et al.*[4] using similar methods claimed the additional presence of melibiose and raffinose, although in a later publication Koch doubted the presence of these two sugars. White & Secor[5] found a fructosyl raffinose in the levosine group of sugars.

The investigation of the sugars in flour has now been repeated and extended using a method similar to that employed by Koch *et al.*[4] Flour (National Scottish Springs blend) was extracted with boiling 70% ethanol and the centrifuged extract was dialysed against distilled water. The dialysable fraction was subjected to quantitative paper chromatography, and the separated sugars after elution from the paper were estimated either directly or after hydrolysis by periodate titration and Roe's colorimetric method for fructose.

A typical qualitative chromatogram is shown in Fig. 1. In this idealized chromatogram a series of slow-moving spots extend from a starting line to a position above that occupied by raffinose. These

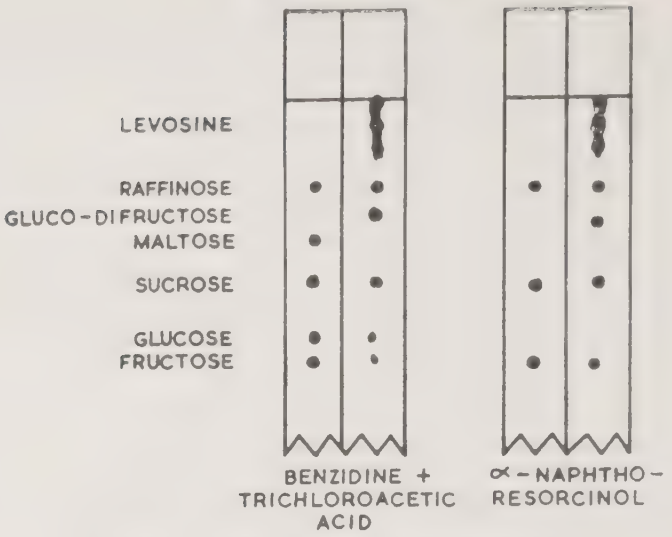


FIG. 1. *Chromatograms of flour extract*

spots were not well separated, but the presence of individual components could be discerned. The component sugars of this fraction contained glucose and fructose, and they were non-reducing. Collectively, they form the fraction generally known as levosine. Strong acid hydrolysis and quantitative investigation indicated that the fructosyl raffinose of White & Secor was present in very small amount, if at all.

Below the levosine group in the chromatogram is raffinose. Separation of this sugar from levosine was not ideal and elution of the raffinose zone of the chromatogram followed by mild hydrolysis yielded traces of glucose. This may have accounted for Koch's doubts as to the identity of the sugar.

Below the raffinose is a non-reducing sugar which on hydrolysis yielded only glucose and fructose in the molecular ratio of 1 : 2. This sugar is similar to the gluco-difructose originally described by Macleod[6] which she found in wheat, barley and rye. It appeared to be a trisaccharide, and although its structure has not been investigated, it is likely to be one of the three possible trisaccharides formed by the addition of a fructosyl radicle to sucrose. These trisaccharides were found by Bacon[7] to arise by fructose transfer and addition to sucrose during the action of the enzyme invertase.

The presence of maltose in flour in more than trace amounts was not demonstrated, probably because the amylase activity was rapidly destroyed under the extraction conditions used.

Table I shows the results of several analyses of the sugars in flour together with an estimation in wheat grain carried out by Macleod &

Table I

Sugars of flour and wheat grain

Wheat grain, g./100g.		Flour, g./100g.				
			Mackenzie & Drummond unpublished	Williams & Bevenue[8]	Colin & Belval[2]	Koch *et al.*[9]
Fructosans	1·030	Levosine	0·45	0·82	0·60	—
Raffinose	0·331	Raffinose	0·17		—	0·07*
Gluco-difructose	0·406	Gluco-difructose	0·33		—	—
		Melibiose	0·0		—	0·18**
Maltose	†	Maltose	†	0·05	—	0·07
Sucrose	0·836	Sucrose	0·27	0·21	0·22	0·10
Glucose	0·092	Glucose	0·04	0·09	0·17	0·01
Fructose	0·057	Fructose				0·02
Total	2·752	Total	1·26	1·17	0·99	—

† Trace, insufficient for estimation
* Unknown, possibly raffinose with some levosine
** Unknown, possibly gluco-difructose

Preece.[8] As already mentioned we found the maltose content of flour to be insufficient for estimation, and the presence of melibiose was not detected. A certain measure of agreement is apparent between the various analyses, in particular the sucrose content quoted by Colin & Belval is very close to those obtained by Williams & Benevue and by the present author. The important fact shown in the last-named is that approximately 75% of the free sugars of flour consist of fructose-containing sugars other than sucrose, i.e., levosine, gluco-difructose, and raffinose. Wheat grain presented a qualitatively similar picture, but the concentration of each sugar was considerably higher than that in flour.

Effect of fermentation on sugars in flour

The changes in sugar concentrations during panary fermentation were followed using a dough containing 2% yeast and 2% salt on the flour and with no added sugar. It was found that the rate of hydrolysis and fermentation of the free flour sugars depended upon the type of baker's yeast employed.

Figure 2 shows typical results of analyses obtained during panary fermentation by two different strains of commercial yeast, denoted as yeast A and yeast B. The widely different substrate available to each yeast can be clearly seen. Fig. 3 shows the changes occurring in the gluco-difructose and combined fructose concentrations. The combined fructose is the total of the fructose contained in the levosine and raffinose fractions. The hydrolysis of both gluco-difructose and combined fructose by yeast B is very much faster than by yeast A. This explains the high fructose concentration available to yeast B in the early stages of fermentation.

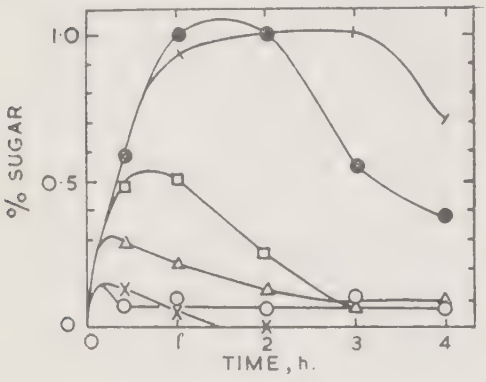


FIG. 1

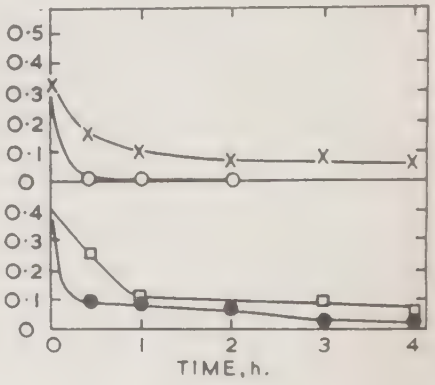


FIG. 2

FIGS. 2. & 3. *Changes in sugar concentrations during panary fermentation*

FIG. 2. Glucose, yeast A ×, yeast B o Fructose, yeast A △, yeast B □
Maltose, yeast A •, yeast B —

FIG. 3. Glucose–difructose, yeast A ×, yeast B o
Combined fructose, yeast A □, yeast B •

Fig. 4 shows the hydrolysis of sucrose which is fast in both cases, and the production of melibiose which is also rapid. This melibiose production is a measure of the rate of fructose transfer from raffinose. Melibiose itself does not appear to be attacked during fermentation.

The over-all production of carbon dioxide during panary fermentation, brought about by yeasts A and B is shown in Fig. 5. These measurements were obtained by means of a Zymotachygraphe which measures gassing rate in ml./min. These dough fermentations were carried out at 27°, using 2% yeast on a flour basis and with no added sugar. The over-all gassing rate with yeast B was higher than that with yeast A and in addition there was no temporary decrease (dip) in the gassing rate at about 1·5 hours.

A number of interesting facts require discussion in Fig. 2. It has been shown by a number of workers, notably Larmour & Brockington,[10] Lanning[11] and Larmour & Bergensteinsson[12] that

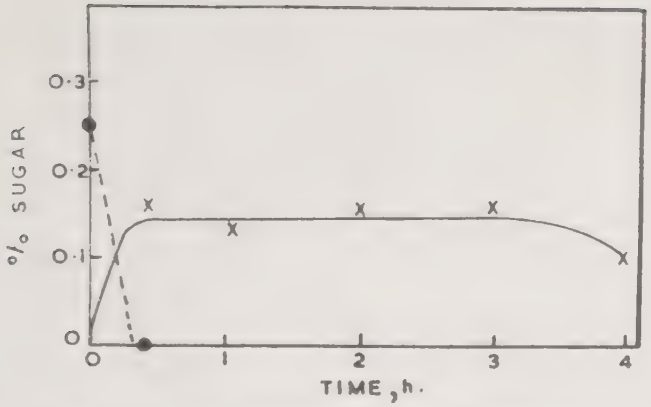


FIG. 4. *Changes in sugar concentration during panary fermentation by yeasts A and B*

sucrose • melibiose ×

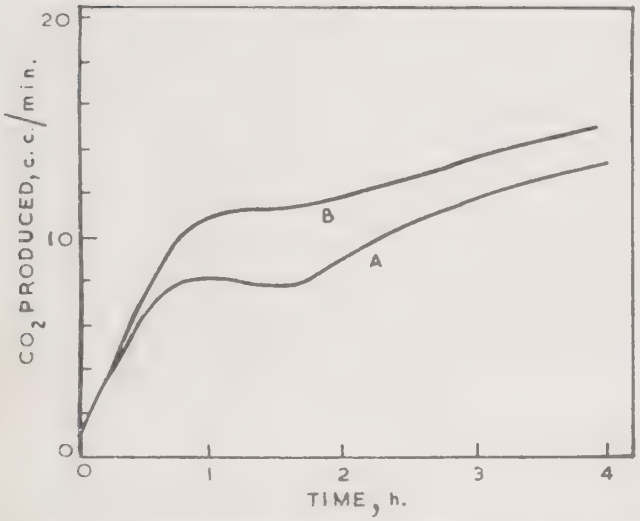


FIG. 5. *Fermentative activity of yeasts A and B in dough*

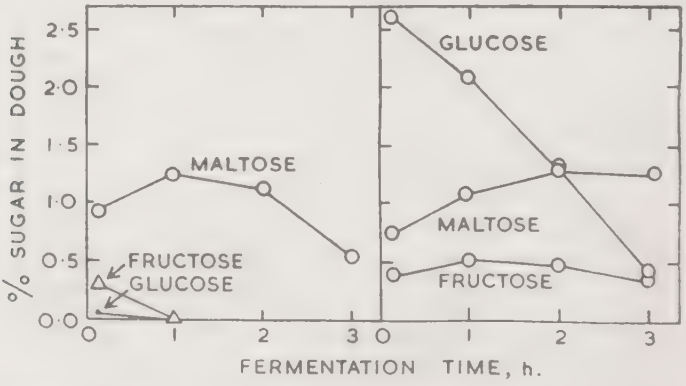
maltose in dough is fermented after the sucrose, glucose and fructose concentrations have reached a low level. This is shown in Fig. 2. Furthermore, as is well known, the glucose is more rapidly consumed than is the fructose.

The sugar concentrations shown in Fig. 2 are residual concentrations, i.e., a measure of the sugar not already fermented by the yeast. Thus the attack on maltose commences after approx. 1·5 hours at which time the glucose and fructose have reached a low concentration, and the changeover to maltose fermentation is made comparatively rapidly. It is well known that certain types of yeast used in baking require an adaptation or induction period before fermenting

maltose, and this fact explains the dip in the gassing rate shown in Fig. 5. Although the onset of maltose fermentation by yeast B probably occurs about the same time, a considerable amount of fructose is still available to the yeast and the changeover appears to be made in a smooth manner and with no temporary reduction in gassing rate. This factor is of some importance in baking.

It has always been held that the invertase content of yeast is not a limiting factor during panary fermentation, and it is certainly true that sucrose is hydrolysed at a considerably faster rate than that at which it is fermented. Nevertheless the rates of hydrolysis of the fructose-containing sugars of the flour exert a profound influence on the nature of the fermentation, and if these sugars are hydrolysed by invertase, the enzyme activity must be a limiting factor in the fermentation, especially in the case of yeast A. The situation with regard to the transferring action of invertase on the fructosans of flour is not clear. Legrand & Lewis[13] claimed to have demonstrated in yeast the presence of an enzyme distinct from invertase which was capable of easily fermenting gluco-fructosans. This claim cannot as yet be supported or denied, but hitherto it has not been possible to separate invertase preparations into substrate-specific components. Such invertase preparations are capable of hydrolysing the gluco-fructosans of flour.

Fig. 6 shows a somewhat similar picture of fermentation obtained by Koch *et al.*[9] who appeared to be employing a yeast exhibiting the properties of the A strain in the previous figure. Fig. 7 taken from the same paper, shows the effect of the addition of glucose at the start of the fermentation. When glucose is added, the fructose and maltose are utilized to a very limited extent. Koch pointed out that 'by



FIGS. 6. & 7. *Changes in sugar concentration during fermentation at* 30° *of a basic straight dough made with* 3% *of yeast* (FIG. 6, left) *with no added sugar* (Fig. 7, right) *with* 5·0% *glucose added* (*Koch* et al.[9])

proper adjustment of glucose and sucrose in the baking formula, fermentation can be accomplished without appreciable loss of fructose from the dough'.

Such sugar additions tend to nullify the differences in enzyme activity with regard to gluco-fructosan hydrolysis as, with addition of sucrose or glucose to the dough, the over-all rate depends solely on the glucose-fermenting ability of the yeast, or on the fructose-fermentation rate which is almost as fast.

While it is difficult to fractionate the various components of the levosine group of sugars, it was possible to isolate by means of a charcoal–Celite column the slowest-moving of the gluco-fructosans. As the levosine sugars contain an average of 1 molecule of glucose to 4 molecules of fructose, it followed that the isolated fraction was likely to contain more than 5 hexose units. One would logically consider this sugar to be the most difficult of the fructosan group to yield to hydrolysis by yeast, and fermentation of this sugar would provide a severe test of the yeast. Fig. 8 shows the fermentation rates of this sugar by three different yeast strains, measured by the usual Warburg technique in a vitamin-salts medium similar to that described by Atkin *et al.*[14] Certain samples of yeast-A type were completely unable to ferment this sugar at a measurable rate. The marked differences in fermentation rate between yeast A and the other two yeasts are greater than those obtained in an actual panary fermentation, i.e., the inability of yeast A to attack this sugar

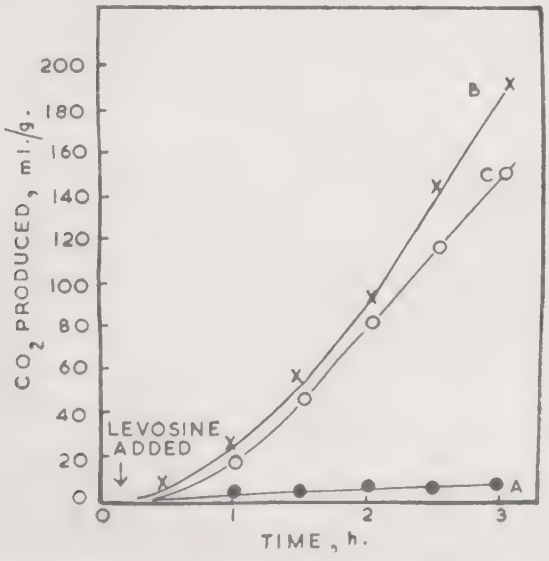


FIG. 8. *Fermentation of levosine (slow moving chromatographic fraction) by yeasts A, B and C*
CO_2 produced at 30° per g. of dry matter

is relative, not absolute. It has already been stated that yeast A is capable of hydrolysing the simpler components of the levosine group, although more slowly than yeast B. The fermentation of this sugar built up to a steady linear rate with no evidence of enzyme induction or yeast growth in the 3-h. period examined. The final rate in the case of yeast B was approximately 80% of the maximum glucose rate of the yeast.

A method of obtaining a measure of the carbon dioxide evolution due to the free sugar components of the flour, as opposed to the maltose produced by amylase activity, was thought to be of interest, and it was finally found that flour amylase was inactivated by autoclaving the dry flour at 15 lb./sq. in. for 2·5 h. This method normally, but not always, produced an amylase-free preparation. (Flour amylase is very heat-stable.) When such a flour was employed in panary fermentation, curves of the type shown in Fig. 9 were obtained. These curves demonstrate the differences already found between yeast A and yeast B and also the fact that the total gas obtained in a normal dough is evolved from free sugars during the early stages of fermentation in spite of the presence of a considerable amount of maltose. Although this method does not provide a direct measure of maltose fermentation, it would appear to be logical to obtain such an estimate by subtracting the curve for free sugar from the total curve. Fermentation curves produced in this way are shown in Fig. 10, where different concentrations of yeast A were added.

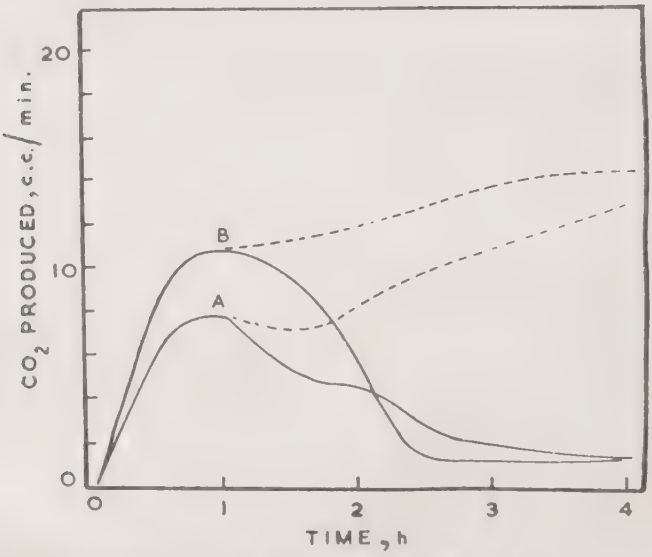


FIG. 9. *Fermentative activity of yeasts A and B in dough prepared from flour in which amylase was inactivated*

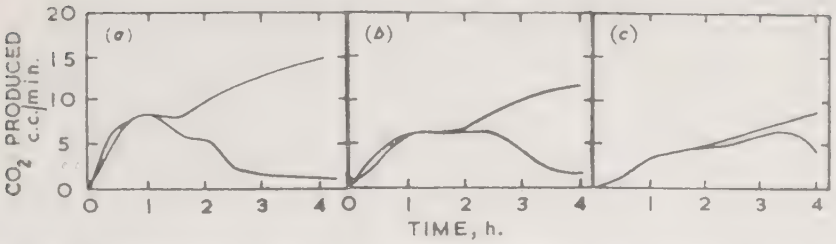


FIG. 10. *Fermentative activity of yeast in normal and amylase-inactivated flours* (*a*) 2·00% *yeast* (*b*) 1·32% *yeast* (*c*) 0·88% *yeast* (*yeast % of flour wt.*)

Application to baking operations

There is a rough method for obtaining the yeast concentration for any given bulk fermentation time in bakery operations:

lb. yeast/sack of flour (280 lb.) = 7·5/bulk fermentation time in h. When this relationship is employed for the concentrations shown, it so happens that the actual proof time corresponds to the period of changeover from fermentation of free sugar to that of maltose. Since there is a constant amount of free sugar available in any given weight of flour, it follows that the lower the yeast concentration employed, the longer will the free sugars persist, and the later will be delayed the onset of maltose fermentation.

Hence in a straight dough fermentation with no added sugar, the bulk fermentation occurs almost entirely at the expense of the free sugar components of the flour, the proof time corresponds to the changeover to maltose fermentation, and only in the later stages of proving does maltose become the main substrate. This appears to hold true regardless of the yeasting level employed, provided that conditions do not depart widely from the formula quoted above.

In conclusion, it may be of some interest to mention that a linear relationship has been obtained between proof times obtained in the experimental bakery and a fermentation rate value obtained by taking the average of the first and third hour instantaneous gassing rates of the yeast measured by the Zymotachygraphe. This was devised on the principle that the rate at the first hour is an estimate of the free-sugar rate, whereas that at the third hour is due to maltose fermentation. (In the bakery a 2-hour bulk fermentation is employed and two different yeasting levels are used.) At the higher of these levels which corresponds closely to the formula already mentioned, a correlation coefficient $r=-0{\cdot}83$ was obtained with $n=168$ yeasts. The yeasts employed were of many different types ranging from very fast to very slow fermenters. At the lower yeasting level a correlation coefficient $r=-0{\cdot}87$ was obtained. Such a low yeast concentration would tend to cause a later changeover to maltose fermentation during the proving period.

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Discussion

In reply to a question as to whether or not the concentration of CO_2 in the dough affects the rate of fermentation, and whether the 'knocking back' affects this rate by removing CO_2, *Dr. H. G. Sykes* said: I can state that 'knocking back' the dough in the zymatograph test has no effect on the rate of gas production, i.e., there is no evidence that fermentation rate in the early stages is restricted by substrate availability, or that the concentration of CO_2 in the dough affects the rate of fermentation.

Mr. R. A. D'Arcy: The omission of salt from panary fermentation accelerates CO_2 production. In the 'delayed salt process' of bread making, would not this omission of salt result in more vigorous fermentation in the initial stages when salt is absent?

Dr. McKenzie: This rapid using of the initial flour sugars would result in general over-all poor production of CO_2 in dough, particularly in the later stages of fermentation—after the salt had been added. This effect would be particularly evident in a flour of poor diastatic activity.

Mr. R. O. V. Lloyd: Has Dr. McKenzie used an aniline–diphenylamine type spray during his paper chromatographic work?

I might point out that this spray is most distinctive for sugars containing maltose linkages. A recent technique (*Science*, 1956, **123**, 543) involving washing the paper with acetone, after development, removes the colours due to maltose-type sugars and reveals traces of other sugars when present. This has been found useful in examination of residual sugars after fermentation of beer and should be applicable to bread fermentation.

Dr. McKenzie: This technique has not been tried.

INFLUENCE OF ENVIRONMENTAL FACTORS ON YEAST BEHAVIOUR

By J. WHITE

(*Fardon's Vinegar Co. Ltd., Birmingham*)

Introduction

It is now generally agreed that, in common with plants and animals and with other micro-organisms, the behaviour of yeast depends upon both hereditary and environmental factors.

Much work has been done and a large literature has been accumulating on the genetical aspects of the subject. Lindegren,[1] for example, has recently stated that there are at least 13 general categories of hereditary yeast characteristics more or less under control, these being:

(1) size of cells
(2) flocculation tendencies
(3) sensitivity to alcohol concentration
(4) sensitivity to salt concentration
(5) sensitivity to sugar concentration
(6) content of respiratory enzymes
(7) resistance to autolysis
(8) colour
(9) sensitivity to cations
(10) ability to synthesise amino-acids
(11) ability to synthesise purines and pyrimidines
(12) ability to synthesise vitamins of the B-group
(13) ability to ferment specific sugars and other carbohydrates

To these should be added:

(14) fermentation rate.[2]

No doubt, as the years go by, other important functions of the cell will be included in the above and work in the genetic field promises one day to produce valuable practical results in the emergence of new yeast strains of scientific and industrial value.

It is believed, however, that equally important results could emerge from a more urgent appraisal of the effect of environment on yeasts and other micro-organisms than has so far been attempted.

It is hoped to give examples to show how many of the basic properties of a given sample of yeast, stated by Lindegren to be genetically controlled, may be radically changed by alterations in the environmental conditions under which the yeasts have been grown. The yeasts produced by these methods of growth may be so

vastly dissimilar in their subsequent properties and behaviour that they might almost be regarded as quite different organisms. In any yeast studies, therefore, it is necessary to state the pre-history of the type organisms used before proceeding to discuss their behaviour in any given set of circumstances. It is this aspect which nowadays makes some of the older studies of somewhat limited value, for merely to state that a brewer's, baker's or distiller's strain has been used for a set of experiments is often quite inadequate.

Brewer's, baker's and distiller's yeasts: non-incrementally cultured growth stages

There are points of great similarity between the many and various yeasts which are used all over the world as brewer's, baker's and distiller's strains. The brewer's and distiller's yeasts are used in the production of alcohol from sugar contained in a variety of substrates and, in their employment, growth is of secondary importance to fermentation. Broadly speaking, in their practical applications about 90% of the sugar in the substrate is fermented into alcohol, the balance of 10% being used in the production of new yeast cells and of traces of by-products, such as glycerol and succinic acid.

In modern industrial processes for mass-culture of baker's yeasts, a single cell of yeast weighing approximately 10^{-10}g. is allowed to multiply vegetatively in increasing volumes of sterilized nutrient wort in laboratory conditions until a few ounces of cells have been grown. These are further multiplied in practical works' conditions by growth in sterile wort in culture vessels of increasing size under the influence of very small, regulated additions of sterile air so that a mass of, say, 1000 lb. of pure culture yeast is produced which should be quite free from contamination with any cells of foreign micro-organisms ('infection').[3] During all this time the yeast cells are steadily multiplying (i.e., assimilating the sugar in the substrate under the influence of the small oxygen potential available) but considerable alcoholic fermentation is taking place at the same time. As a result, the yield of yeast obtained (wet; 27% solids) is very small—perhaps no more than 10–30% calculated as a percentage of the sugar decomposed. The balance of the sugar is converted into ethyl alcohol, some of which is lost to the system by volatilization in the small stream of air used. Yeast produced by growth in this manner has been termed 'non-incremental' and the products in brewer's and distiller's processes are similar in nature – that is, they have been produced under environmental conditions which allow for assimilation and alcoholic fermentation to take place concurrently by placing the initial seed into a wort of significant sugar concentration.

Baker's yeasts: the incremental stage yeasts

Having produced several hundredweights of pure seed yeast under non-incremental conditions the yeast manufacturer proceeds to multiply the yeast under far more economic conditions in which wasteful production of alcohol is suppressed and maximum growth or assimilation is encouraged. This is done by suspending a specific quantity of seed yeast into a large bulk of water containing inorganic salts (e.g. sources of phosphate and sometimes magnesium and ammonium salts) and gradually adding the source of sugar, e.g., a solution of molasses together with other inorganic nutrients (notably ammonium salts) in small increments over the course of the assimilation which may last several hours. During all this time a great volume of air is passed through the suspension. Under the correct conditions, alcoholic fermentation is entirely suppressed and all the sugar is converted by assimilation into the new yeast cells, at such a high assimilation efficiency that the yield may be as high as 210% of yeast (of 27% solids content) calculated on the sugar used (as invert). In such manner 10 cwt. of yeast seed may be multiplied to yield 2 or 3 tons of yeast after, say, 11 hours of 'incremental' or 'trickle' feeding. Yeast produced in this manner is now called 'incremental' yeast to denote the nature of its development, which is essentially growth without alcoholic fermentation at extremely small sugar concentration (often no more than 0·0004% w/v). In manufacturing practice, such yeast may be used as seed for yet a further stage—indeed the number of development stages is limited in practice by the necessity to produce a final crop of yeast which is not too highly infected with foreign micro-organisms, which could ruin its storage properties.

During the last few years it has gradually been realized that the environmental conditions of growth produce considerable differences in behaviour between incremental and non-incremental yeasts.[4] When placed into sugar solutions containing nutrient salts, incremental yeasts normally pass through a period of considerable cellular stress and change in the early part of the ensuing fermentation process before they stabilize themselves and reach equilibrium with their surroundings. A fermentation of this type can be divided into three separate phases (see Fig. 1; Curve 'A'). There is first a loss phase in which a loss in cell dry matter occurs during the first few minutes proportional to the osmotic concentration of the surrounding medium. (*Note.* Sugar usage with CO_2 evolution is taking place during this phase.) This is followed by a recovery phase in which the cells gradually overcome the effect of immersion in the medium and new growth (dry matter formation) begins, gradually at first and

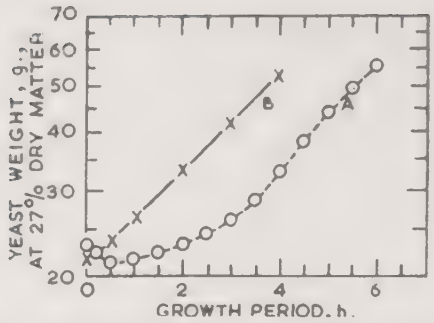


FIG. 1. *Typical curves illustrating the growth of incremental and non-incremental type seeding yeasts when placed in* 20% *(w/v) beet molasses wort (containing nutrient salts) at* 30° *with steady aeration*
(semi-logarithmic scales)
Curve A: first stage, incremental yeast seed. Curve B: second stage, non-incremental seed

then at an increasing rate. Finally, when equilibrium with the surroundings has been achieved, the truly exponential phase begins, and this is ended only at the point when the sugar in the substrate is exhausted or when the products of the fermentation tend to produce inhibition. The time lag elapsing before the exponential phase is attained depends on the concentration of the nutrient solution, and if this is too high the exponential phase may never be reached. The precise growth rate during the period of the exponential phase also depends on the wort concentration, but is particularly influenced by the seeding rate. As the yeast seeding rate is increased, the value of the growth rate is reduced, particularly at very high seeding rates.[5]

The lengths of the initial loss phase and of the recovery phase are very greatly influenced by the seeding rate. Small seeding rates in worts of average concentration give rise to comparatively short recovery phases, whereas with large seeding rates the recovery phases may be so prolonged that the sugar in the substrate may be used before the exponential growth phase can be reached—that is, there is too much yeast present to be 'conditioned' or 'adapted' and the sugar may be used up before full adaption has taken place.

When non-incremental (direct culture) yeasts are used for seeding purposes, however, a very different state exists. If cultivated initially in wort of strength comparable to the wort in which the seed yeast is to be placed, there is no loss- or recovery-phase, and growth takes place entirely exponentially, whatever the seeding rate employed (see Fig. 1; Curve 'B'). The precise growth behaviour of non-incremental yeasts depends on the relative osmotic concentration in which the yeast is primarily cultured and the concentration into which it is finally seeded. Thus, a non-incremental yeast cultured initially in a comparatively low concentration may pass through

slight loss- and recovery-phases if placed into a wort of considerably higher concentration than that of the original culture medium.

Non-incremental yeasts normally give greater rates of total sugar usage than incremental yeasts when placed in a medium of moderate or high concentration. The old classical conception in the literature of 'lag' phase in which no growth was found to have occurred for several hours after the addition of yeasts to the wort could be explained on a loss- and recovery-phase basis, the algebraic sum of loss and growth after some hours being nil: this gave the impression that no significant change had taken place over the period in question.

Sensitivity of yeasts to salt, sugar and alcohol concentrations

It is obvious that all yeasts are affected by the osmotic pressure of the solution in which they are suspended and that, as the osmotic pressure increases, there comes a time when the fermentative activities of the yeast will first be diminished, and finally prevented, by the osmotic concentration of the substrate. Thus, osmophilic yeasts are organisms which will ferment sugar in media of extremely high sugar concentration.

Most normal brewer's and baker's yeast strains are prevented from fermentation by very high osmotic pressures and will not produce gas from media containing more than about 50% (w/v) of glucose.

Some baker's yeasts have been examined which are extremely sensitive to the effect of the osmotic concentration of the medium when tested in dough; these have been termed 'osmosensitive' yeasts to distinguish them from the normal commercial (incrementally-cultured) baker's yeasts which exhibit the minimum of sensitivity towards the osmotic effects ('osmosensitivity' is a relative term).[6, 7] The author's interests in this matter were aroused some years ago when a non-incrementally grown yeast which possessed remarkably fine gas-producing properties in sugar solutions was tried commercially in a bakery. The yeast fermented white bread dough 'wonderfully well' (to quote the practical baker), but was a complete failure when used the same day to make wholemeal bread the dough of which produced hardly any gas and gave very small loaves on baking. Subsequent work showed that the yeast was extremely osmosensitive. Its gas-producing rate was drastically reduced when the normal salt usage rate of 5 lb. per sack of flour (of 280 lb.) (in white bread) was increased to 6 lb. in the wholemeal bread, and this osmosensitivity was still further aggravated by the presence in the wholemeal flour of a potent fermentation inhibitor which was particularly active against the osmosensitive yeast at the higher salt concentration (see Fig. 2).

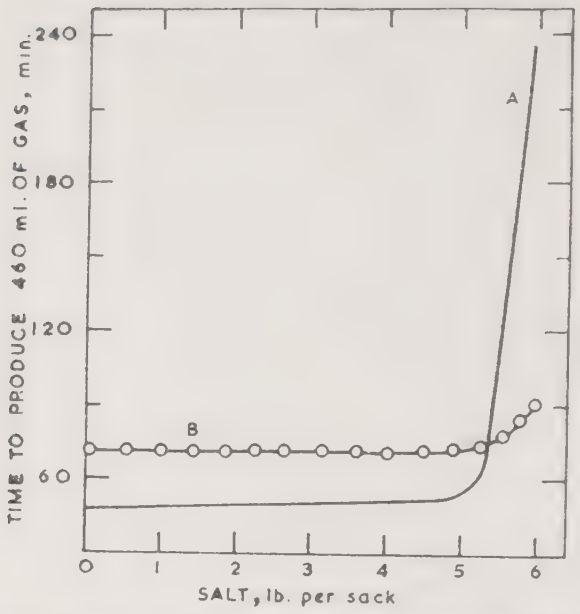


FIG. 2. *Dough fermenting activity of* (A) *osmosensitive yeast* (B) *normal yeast* (Fermentation activity expressed as a function of time required for a standard volume of gas to be produced in test)

Yeasts of the incrementally fed type propagated in exponentially fed fermentations over several stages are, when fresh, very little affected by the fermentation inhibitors in the flour even under conditions of comparatively high osmotic pressure. Such yeasts normally produce gas at satisfactory rates when tested in doughs or sugar solutions of moderately high concentrations. When these yeasts are seeded into an aerated sugar solution under conditions in which growth and fermentation take place simultaneously, considerable changes take place in their nature. Any yeast crop grown in this manner becomes less stable to the effects of osmotic pressure and fermentation inhibitors present in flour, i.e., the yeast becomes osmosensitive. The degree of osmosensitivity induced has been shown to increase as the growth rate of the yeast increases. Thus, yeast crops separated at the height of growth activity, i.e. in the logarithmic phase of growth, are very osmosensitive and this condition is accompanied by very poor keeping quality. Such yeasts rapidly deteriorate in fermentative activity with storage, and rapid autolysis always appears to accompany the condition of osmosensitivity. A degree of stability is given by allowing such yeasts to complete their fermentation cycles, comparative stability being reached in the last few minutes of fermentation, as the sugar in the substrate becomes rapidly exhausted (Fig. 3). Growth rate has been

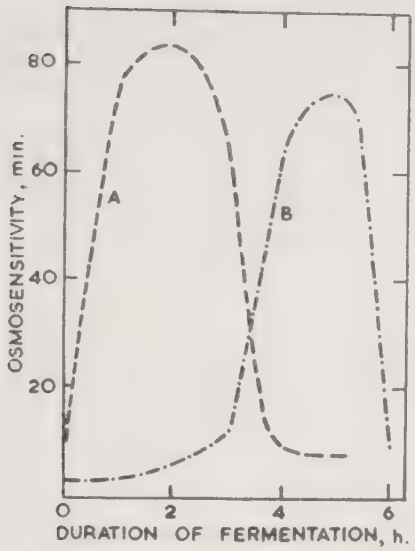


FIG. 3. *Production of osmosensitivity in yeast by growth in sugar solution (20% molasses wort)*

Note production of osmosensitivity in phase of rapid growth. A. using non-incremental seed yeast. B. using incremental seed yeast

experimentally modified by variations of temperature and aeration, but osmosensitivity of the crop has always varied with the yeast growth rate under all conditions using a variety of substrates, e.g., malt wort, molasses worts, glucose, sucrose, invert sugar and maltose solutions.

Yeasts grown under non-incremental conditions (i.e. when growth is accompanied by fermentation) develop osmosensitivity on storage faster than do incrementally-fed yeasts, the increase being greater as the storage temperature is raised. Yeast crops harvested at the height of rapid growth activity are most unstable when stored and may autolyse within a few days of storage at 20°. Crops from fermentations which have been subjected to low aeration have been found to be sticky in consistency and subject to very rapid autolysis when stored at 20–30°. Solutions of salts, sugars and alcohol all reduced gas production in proportion to the osmotic pressure given by the additions of such substances. Sensitivity was thus shown to sugar, salts and alcohol as a function of osmotic pressure, not of chemical constitution of the added substance.

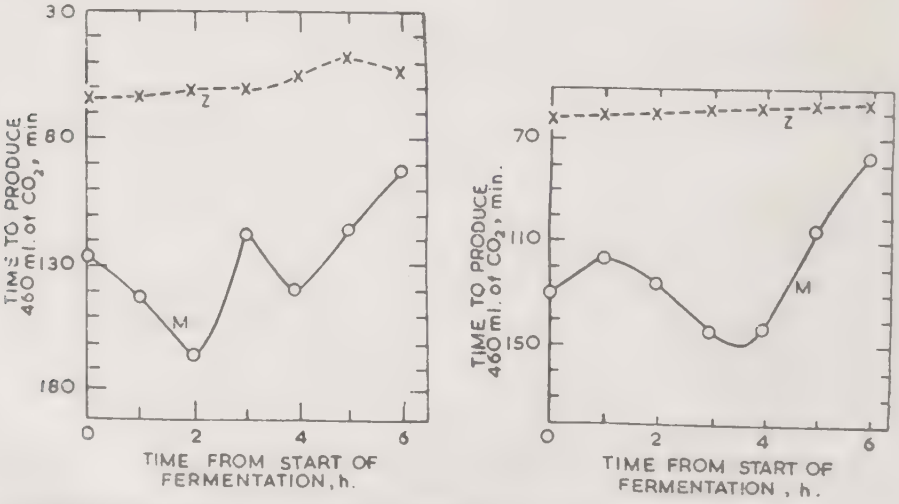
Production of enzymes by yeast as an adaptive process

To have a high rate of fermentative activity in bread doughs it has been shown that a yeast must possess a high content of the zymase and maltozymase enzyme systems (i.e. ability to produce carbon dioxide from glucose and maltose, respectively).

Enzyme production in yeasts by adaptation of the organisms to growth and fermentation in a medium containing the appropriate sugar source is a commonly accepted phenomenon. For example, yeasts grown in maltose solutions develop high maltozymase activities, whilst immersion of yeast in galactose solution produces a galactose-splitting enzyme system, albeit in the latter case many hours of contact with the sugar are necessary before any fermentation of sugar occurs.

Production of maltozymase in a (baker's) yeast strain initially with only moderate activity of this enzyme is so effectively achieved by growth and fermentation of the yeast in maltose solution (containing nutrient salts and vitamins) that yeasts so treated may ferment maltose at a somewhat greater rate than glucose.

Production of maltozymase activity, however, is by no means confined to adaptive mechanisms by residence of the yeast in maltose solutions. It has been found that the maltozymase activity of yeast can in some cases actually be increased by growth in sucrose, D-glucose, invert sugar and beet molasses solutions, although initial contact of the yeast with these sugar solutions produces a sharp reduction of maltozymase activity in all cases. Maltozymase is obviously a constitutive enzyme system in the yeast strain used and in addition to its elaboration by adaptive mechanisms it appears that the enzyme system is formed at certain stages of the fermentation and growth cycle of the yeast by mechanisms which are characteristic of the yeast cell itself, whatever the substrate used[8] (Figs. 4 and 5).



Zymase (Z) *and maltozymase* (M) *activities of yeast crops harvested at intervals during* (Fig. 4) *sucrose and* (Fig. 5) *glucose fermentation*

The zymase content of the yeast cell is increased as the rate of sugar decomposition increases: conditions which lead to high rates of sugar usage by yeast growing and fermenting in a sugar solution (whatever the chemical nature of the sugar) produce increases of zymase in the yeast cells used.

Invertase is produced in large quantities in yeast by growth and fermentation in sucrose solutions so long as any sucrose is left in the solution. As soon as the sucrose is completely inverted, the invertase content of the yeast crop is reduced very rapidly. Invertase, however, is obviously a constitutive enzyme in yeast, for it is present in the yeast after repeated growth in non-sucrose-containing substrates. When the yeast strain used in this work was placed into maltose solution its invertase content was actually increased over the first hour's growth, although further growth produced some reductions in invertase content.

This work on enzyme production emphasized that, when yeast is placed into a sugar solution under conditions in which growth and fermentation take place, instability is produced in the yeast, ultimate stability (particularly with regard to its maltozymase system) being reached when most of the sugar in the medium has been used. Similar instability was found in studies on yeast osmosensitivity.

As a biological system, yeast is vitally changed by immersion in sugar solutions and yeast properties are functions of the composition of the nutrient solutions and also of the actual stage throughout the fermentation at which the yeast is harvested. A yeast fermentation is thus seen to be a cycle involving considerable dynamic changes in yeast composition.

Cell size

Whilst cell size is stated by Lindegren to be governed by genetical factors, the influence of environmental conditions on cell size is obvious on examination of yeast cells throughout their growth through non-incremental culture stages into the final incrementally-fed stages in a compressed yeast plant. The initial cells in the culture stages may be 12μ or more in diameter, diminishing to 6–4μ and even less in the final commercial stages after incremental feeding.

Cell water content

There is considerable evidence to demonstrate the influence which environment has upon the natural water content of the yeast cell. It has, for example, been shown that the temperature at which the yeast is grown has a major effect on the moisture content of the cells constituting the yeast crop.[9] In one series of experiments it was

shown that at 20° the cells of the crop contained on the average 76·2% of water, whereas cells grown at 40° contained only 68%. It was also shown that cells cultured in a synthetic medium contained more water than cells grown in a medium of similar composition but presenting the sugar as molasses not as sucrose. A further reduction in cell water content was produced when the molasses was fed into the solution incrementally (exponentially).

Perhaps the most interesting fact emerging from this particular set of studies was that repeated sub-culturing of yeast in a high-gravity malt wort resulted in reduced water content of the cells derived from the culture grown in this manner. A yeast culture was maintained in malt wort of specific gravity 1·059 and about 10% of the liquid culture was inoculated twice weekly into a fresh flask of sterile wort. A careful check of the water content of the cells of the yeast produced from such cultures was kept. The water content of the cells from the original culture was 68·8% and this was reduced to 64·2% after 60 sub-cultures had been made, this figure being further reduced to 61·2% after 90 sub-cultures. This reduction in cell water content was found in all yeasts made from these sub-cultures, even when made in ton-quantities on the commercial scale. This was a matter of considerable economic importance, as the yield of pressed yeast diminished as the water content of the cells comprising the yeast mass was reduced. As the changes were found to persist throughout all yeast crops grown from the individual sub-cultures it was impossible to escape the conclusion that the water content of the cells may be controlled by some hereditary mechanism which in its turn was subject to gradual and regulated change by the environmental influence of the sub-culturing technique employed. This pattern of behaviour was repeatedly observed over many years using several distinctly different single-cell yeast cultures. This appears to be an example of the permanent effect of environment on yeast properties.

Conclusion

It is hoped that examples have been given which demonstrate how important environmental conditions can be to certain properties of yeasts. Many of these properties will be seen to be those which Lindegren has stated to be subject to genetical control. There would appear, therefore, to be an urgent need to integrate the genetic and environmental studies to understand fully and to predict how any yeast will behave under any stated set of circumstances. This could set the pattern for better comprehension of the behaviour of the large number of micro-organisms which are daily becoming more and more of industrial importance.

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Discussion

Dr. C. Rainbow: There is evidence (C. Rainbow, unpublished) that certain beer-spoilage lactobacilli possess a mechanism whereby maltose is fermented directly, i.e., without the hydrolytic intervention of maltase. Dr. White's experiments suggest that yeast, too, might possess such a mechanism. Is there any direct enzymic evidence that this is so?

Dr. White: I have no evidence of such a mechanism.

Dr. H. Holzer: (1) Respiration and fermentation—have these been measured under different growth stages for types A and B?

(2) How large are the differences of fermentation between glucose and maltose?

Dr. White: (1) *Total* gas production has been studied but not *divided* into gas due to *respiration* and *fermentation*.

(2) About 10% or sometimes rather more.

Mr. J. S. Harrison: In reply to Dr. Rainbow's question, and the comment by Dr. Holzer, Mr. Burrows at Epsom investigated the haploid yeast strain (mentioned in Mr. Fowell's paper above) which fermented maltose more rapidly (about 30% faster) than it fermented glucose. No evidence of the participation of phosphate in this process was obtained either by *'in vitro'* or *'in vivo'* experiments. Although this does not disprove the involvement of phosphate, it is possible that the method of breakdown of maltose might provide a form of glucose which is more reactive than the normal product.

Mr. G. Loggers: What were the maltose and glucose concentrations in the experiments in which maltose was fermented more rapidly than glucose.

Dr. White: They were equal.

Dr. H. Jørgensen: Can Dr. White say something as to the nature of the inhibitory substance in flour?

Dr. White: The inhibitory substances were fat-soluble. They may be similar in nature to the sulphur-containing peptide isolated in the U.S.A. by Balls and co-workers.

Questioner: The yeast fermentation poison referred to by Dr. Jørgensen may have been extraneous to flour. Some years ago we met a wholemeal flour which would not ferment on doughing; this was due to accidental Gammexane contamination. This substance acts as an inositol antagonist.

Dr. White: The inhibition mentioned has been found in very many samples of flour, invariably more in wholemeal flours than in treated ('whiter') flours.

SIXTH SESSION

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Chairman: SIR R. H. V. LEVINGE

GENERAL DISCUSSION

Dr. A. K. Mills: Oleic acid can replace biotin as a growth factor for certain bacteria, and it is considered that it facilitates the entrance of biotin into the bacterial cell. I would ask Prof. Conway to comment on this with relation to his own theory of the cell wall in general, and whether he would expect the same sort of mechanism to be found in the yeast cell wall.

Prof. E. J. Conway: We have no direct experience on that, and I have no information that might be of value. The membrane across which the transfer is occurring is undoubtedly largely lipid, largely made up of lipoid–protein layers but how oleic acid can alter that significantly to facilitate the entrance of biotin I really cannot say.

Prof. R. H. Hopkins: Following Dr. Mills' first question, is not the effect of oleic acid the same as the more general effect of fats in media in which growth factors are being determined by microbiological methods? E.g., using lactobacilli for determination of riboflavin in grain, the grain must first be defatted or high results will be obtained.

Dr. A. K. Mills: I think the whole thing is connected. It has been found that with certain bacteria you can decrease the amount of biotin and add oleic acid and obtain the same growth result. It was first considered that it may be a growth factor but may merely facilitate the entrance of biotin into the cell.

Dr. H. Holzer: I was very astonished at the effect of animal hormones on yeast, the biochemical action of which on animals are not known. Are there any other experiments with other hormones, e.g., insulin or hexokinase? From experiments with animals one might think that there is an effect on the glucose uptake connected with the glucose phosphorylation in animals.

Prof. E. J. Conway: With regard to the effect on animals of phosphorylation of carbohydrate, this was investigated many years ago and found to have a completely negative result. We get this inhibition of uptake of potassium also with cortisone.

Dr. F. Winder: In previous discussion, it was stated that Lindegren has claimed that inorganic polyphosphate accumulates on the chromosomes of yeast before nuclear division and suggested that polyphosphate hence plays a special part in DNA synthesis. This is not in keeping with current views on polyphosphate functions. It is usually held that polyphosphate is only a reservoir of phosphate and perhaps energy, the phosphate in it being capable of being drawn into the active phosphate pool through reaction with ADP to form ATP. This also appears to be the case in mycobacteria. Using ^{32}P we have shown that cell polyphosphate is not a direct precursor of any of the major 'stable' cell phosphorus compounds, nucleic

acids, phospholipid, etc. Lindegren's observation is the only one I know which conflicts with this view. I gather that his views on the question are not accepted by many other cytologists, such as Mudd and his school. Could some cytologist here provide an alternative explanation of what Lindegren believed to be an observation of polyphosphate accumulation on chromosomes?

Dr. H. Holzer: I think that polyphosphates are not for storage of energy but are only the reservoirs of phosphate in the cell.

Mr. D. H. F. Clayson: I wonder whether Dr. White uses the right term, 'osmosensitivity'. It seems to me that what he has described involves the exposure of the yeast cell to high osmotic pressure and that in fact would not be a tendency for the yeast cell to burst. Is it possible that an increase in the salt concentration produces a corresponding adverse effect on the internal pH of the yeast cell under certain cultural conditions?

Dr. J. White: What does happen to these cells? They are perfectly healthy cells in the ordinary way; I might almost say, too healthy. They have been growing at least under correct conditions, give tremendous fermentative activity, much greater than average, incrementally fed baker's yeast, but when subject to these conditions of high osmotic pressure, whether given by sugars or salts, they just do not produce the gas. What the mechanism is I should be very interested to know. It might possibly have something to do with the content of respiratory enzymes. The question of the cell wall having been damaged is another possible explanation. I have no evidence one way or the other.

Prof. E. J. Conway: I think a very small amount of work has been done on the yeast membrane. If you put yeast cells into increasing osmotic pressure solutions, you get quite a marked loss of water. If there are differences of elasticity, you can get very big differences in the water losses. The osmotic pressure of the fluid inside the yeast cell is surprisingly high. Normally if you bruise yeast cells in liquid or in oxygen you release their contents and then, if you take the depression of the freezing point and interpret in terms of molarity, the value obtained is something like 0·5 molar, a surprisingly high figure. This would appear to indicate that the same pressures are inside as outside the cell membrane at approximately 18% of sucrose, which is very high. If the elasticity of the yeast cell wall were more investigated, quite interesting results would be obtained in relation to such questions. It is possible indeed that Dr. White has done such experiments himself.

Dr. J. White: Prof. Conway has reminded me of some work done a long time ago. We determined the loss of water in the various salt

and sugar solutions and found substantially no difference—in fact, incrementally fed yeast appeared to have the same osmotic pressure as yeast walls grown at 10–20% solution. We did find that an extremely small salt concentration withdrew a large amount of water from the cell. I personally do not regard the internal pressure of the cell as being as high as Prof. Conway suggests because at as little as 0·3% NaCl you can prove quite successfully that water has been withdrawn from the cell.

Determination of osmotic pressure by looking to see whether shrinkage of the cell has taken place only shows a gross withdrawal of salt water. Much water is withdrawn from the yeast cell with very little salt. My own feeling is that very small concentrations of salt outside the yeast cell will produce withdrawals of water.

Prof. E. J. Conway: One cannot have really firm conclusions on that until one has measured the freezing point depression of the cell. Have you done depression of the freezing point?

Dr. J. White: No. Some years ago I carried out experiments in which I took a number of yeasts and mixed each with a standard quantity of water and determined its viscosity. On addition of very small amounts of salt to the suspension, I found that the viscosity had been reduced due to withdrawal of water from the yeast cells.

Prof. E. J. Conway: The only direct way in which you could confirm this would be freezing point depression. I cannot think of any other direct way.

Dr. J. White: Much more work will have to be done to study how the changes come about. I do not believe in the classical conception of what happens to yeast when it is placed into a solution of salts or sugars. Withdrawal of water from the yeast cell cannot be followed by microscopical methods.

Prof. E. J. Conway: You must compare with a standard yeast cell in a standard way. It is not sufficient to say that you withdraw water from the cell due merely to concentration of salt outside. What are you comparing it with? Are you comparing it with yeast cells suspended in water? These conclusions would not point to the real level of osmotic pressure inside. I think you will agree that very often the level of potassium in cells is 150 mM. If we take the water which is inside the cell out of the chamber, that must multiply the concentration by a factor of a value up to two.

Dr. A. A. Eddy: Very high osmotic pressure inside the cell may be balanced by the elasticity of the cell wall. If you change the pressure outside this cell then you will get a change in the size of the cell. Very little pressure outside the cell may affect the movement of water from the cell.

SUMMARY FROM THE POINT OF VIEW OF THE BREWING INDUSTRY

By A. H. COOK

(*Brewing Industry's Research Foundation*)

I would like this review to take the form of a general body of remarks which I would like to 'sandwich' between two over-all impressions. My first impression, which I hope may be yours as well, is one of wonder at the magnificent way in which this symposium reflects, more than any other that has been held before, the long overdue upsurge of research on yeast. Looking back, one perceives that the principles of bacteriological classification were laid down many years – even decades – ago, doubtless because of medical implications of the subject. Parallel principles of yeast classification only appeared, however, in quite recent years and still give rise to controversy. The reasons may be that in yeast classification a relatively small part is played by morphological study and a large part by the use of biochemical characteristics, in distinguishing between species and strains.

In the past, that rôle has been restricted because the biochemistry of yeast, as we have heard, has been concerned, and is concerned, with molecules with which the chemist has had very inadequate tools to deal.

We have heard for instance mention from Dr. Slonimski of nucleic acid, which, together with inositol, may be concerned with the synthesis of fats as described by Dr. Challinor. Dr. Jørgensen has mentioned mannans and Dr. Eddy in the same context the presence of strongly resistant glucans in the yeast cell-wall; Prof. Hopkins drew attention to both an enzyme which hydrolyses the α-1:6-linkages, occurring in brewing yeast, and to the special glucamylase in *S. diastaticus*. Mention of any of these entities would have been quite impossible a few years ago. I think if we could look at the glossary that Dr. Slonimski said he was compiling of the terms used in this symposium, certainly 20 years ago and even 10 years ago many of them had not been invented for the chemist's vocabulary.

What has all this got to do with brewing? I suggest that we can attain a balanced view if we regard all this new information as falling into two categories. On the one hand there are new facts involving individual strains and species. On the other there are the observations concerned with mutual effects of one strain on another, or one species on another. The brewer in Great Britain at least commonly employs mixtures of strains in his brewery and no one from any immediate scientific standpoint would wish him to do

otherwise. It is when difficulties arise, for example, in apparently changing flocculation and attenuation characteristics, that one needs to know about the composition of the mixture and the contribution of individual components and so in that way to approach the problem of controlling undesired change.

Dr. Eddy contributed penetrating observations on three topics: (1) the inherent distinction between top-fermentation and bottom-fermentation character; (2) flocculence; and (3) finings behaviour. Of these three, the most important is perhaps flocculence, and here we see new conceptions emerging which make it appear that this phenomenon is a property connected, not as hitherto supposed, with a single factor, but with a whole range of factors which can affect the flocculent behaviour of any particular strain. Non-flocculent and self-flocculent types have been observed, and there has also emerged a new conception of mutually-flocculent yeast types. These results appear to have a very definite and immediate application to the brewer's work. I suggest that they give the brewer a new insight into his yeast, and thus, one would think, must enable him to detect and possibly remedy, changes in yeast flocculence, if not indeed avoid them altogether. Much of the difficulty arises from changes in the composition of the yeast mixtures. The precise reasons for these changes remain very difficult to understand and a full discussion of this subject would take us far from the immediate topics of this symposium. Some may ultimately be traced, however, to trifling changes in the wort in relation to requirements of one or other of the constituents and here inositol, ergosterol, general hydrophobic substances and others have been mentioned in connexion with Dr. Challinor's and Dr. Slonimski's papers.

Coming briefly to finings behaviour, this was revealed as being due to the existence on the yeast cell under operating conditions of an electro-negative charge which is neutralized by the electro-positive charge carried by the finings particles. Put briefly, these findings tell the brewer how to ascertain when in a particular instance fining is possible and when it is impossible. Moreover, we are beginning to understand how and under what conditions this necessary negative charge is best developed on the yeast cell. In addition, we heard how a structure of parallel fibres in the finings is necessary in order to have the desirable finings property. Clearly, we are approaching the position when finings difficulties can be eliminated and even permanently removed.

Dr. Slonimski's paper, although it had to do with baker's yeast, had its lessons for brewers as well. That paper drew attention to the hitherto unrecognized and still puzzling ability of certain mutant

yeasts at least to grow anaerobically without normal enzyme synthesis and this he said they do in the absence of glucose by drawing on an amino-acid pool resulting perhaps from the breakdown of nucleic acids. The demonstration by Dr. Challinor of the fragility of the yeast cell under certain conditions taken in conjunction with recent reports on the development of new yeast strains following such fragmentation, may, remembering the difficulties of storage and maintenance of yeast, be worthy of study in the light of this new knowledge.

Prof. Hopkins' paper was notable for its very modest statements as when, for example, he said of the glucamylase of *S. diastaticus* 'it might be present with disadvantage or it might be present with advantage.' It seems, indeed, that there is a considerable potential importance to the brewer in so controlling the degree of attenuation. It appears to offer a purposeful way of bringing about a desired degree of super-attentuation and so obtaining beer more suitable for diabetics because of a reduced carbohydrate content.

Turning to the second category of observations, namely, those dealing with mixed yeasts or mixed microbial populations, Dr. Eddy's method of quantitatively estimating wild yeast in cultures of brewer's yeast deserves mention. The regularity with which quite small populations of wild yeast grow selectively on a medium, which depends for practical purposes on lysine as the sole source of nitrogen, is quite remarkable. Here again the method is one offering immediate practical value to the brewer in enabling him to recognize any trend towards undue increase in wild yeast population before the increase makes itself felt as a difficulty in the brewery.

Mr. Beech's paper was of very considerable interest from the brewing point of view. In it he described the fluctuating composition of organisms present during cider fermentation. The reasons for this fluctuation will probably remain obscure for some time to come but one tends to speculate on what these reasons might be. Possibly trifling changes in the constituents of the medium and competition for them among the different strains may go some way toward any ultimate explanation.

Much of the discussion bristled with ATP, ADP and other abbreviations which must have been unfamiliar to many brewers. The scientist often discerns a possible bearing of these conceptions on fermentation problems, but mostly it is not yet possible to reduce them to the level of day-to-day practical problems.

Mr. Beech's paper was in some respects paralleled from the brewers' point of view by Dr. White's contribution which also dealt with mixed cultures and more particularly the effects of environ-

mental conditions on a whole range of factors which, taken together, must be important to the brewer. Here again we are admittedly a long way from applying such results to specific problems, but the basic knowledge is undoubtedly beginning to appear even if additional work on the part of individual breweries is quite inevitable.

This brings me to the last general impression which I, at least, take away from this symposium. Mr. Gilliland said that the brewing scientist endeavours to do more than find scientific reasons to justify empirical knowledge. How true he was. At the same time he said some of his efforts in the genetical field were perhaps premature because the brewer could rarely define what was wanted or hoped for in a hybrid brewing yeast. Perhaps, however, Mr. Gilliland is already to some extent talking in the past. There are objective methods coming forward, for example, for measuring yeast attenuation and other characters dependent on the yeast as well as further properties of beer associated with various other factors. On that score there is progressively less and less anxiety, while the results reported are undoubtedly fundamental to our understanding of many of these beer characters. The question is, can these results be referred at once to the brewer and here I think we must admit there is, despite these very encouraging advances, still a very wide gap between translating these findings, many of them necessarily obtained with individual strains of yeast, into terms of practical reality in the individual brewery.

Now the brewers here do not need me to tell them that there is no unanimity in the brewing world as to the need of research of the kind we have been hearing about. We ourselves may be quite sure of the need for it, but I am not so certain that there is a unanimous view in the industry at large. Nevertheless, it is inevitable that, as more and more results emerge, such as those in this Symposium, they must be paralleled by an increasing bulk of work in individual breweries. Circumstances in individual breweries are never exactly parallel, even in neighbouring breweries, and there must accordingly be a corresponding bulk of applied work carried on in individual breweries. Having heard, for instance, of the multiplicity of factors which may bear for example, on flocculence, how can the brewer know which actually affect his yeast without doing work to this end on the spot? This is not necessarily new but the Symposium has brought it afresh before us. If the boardrooms can be convinced of the increasing need for applied research to utilize these fundamental results, the Symposium will, indeed, have made the greatest possible impact on the brewing industry.

SUMMARY FROM THE POINT OF VIEW OF THE BAKING INDUSTRY

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Although four of the papers read at this Symposium do not deal with the use of yeast in baking, a symposium such as this does much good, because from the papers as a whole, one realizes that there are many common points or cross-linkages between beer and bread, and not a few of those points can materially contribute to knowledge of the whole topic of fermentation, so that summarizing on behalf of the baking industry is not so easy as appears at first sight.

For example, when listening to Mr. Beech's paper on cider fermentation it occurred to me that reading about the control of bacteria in panary fermentation by the addition of acetic acid, the common method of avoiding 'rope' formation due to *B. subtilis* growth, could have led to forecasting the role of sulphite in achieving control over bacterial contamination in cider. In the opposite direction of thought, was there anything the cereal scientist could derive from the papers by Dr. Challinor and his colleagues and by Prof. Hopkins? Ignoring the difference between brewer's yeast and baker's yeast, were there not thoughts induced by these papers when one remembered the particular softness of bread naturally apparent in long panary fermentation processes, and in the modern shorter processes artificially induced by the addition of fatty crumb softening agents? Were there important clues in the observations on the effect of inositol deficiency on fat formation in the cell described by Dr. Challinor and what will the nature of these fats prove to be? How much cellular destruction is required, before they become freely available for specific actions associated with the starch in bread? Do similar things occur, not necessarily for the same reasons, in long-process panary fermentation, and was the softness problem from the lipid point of view also linked with Prof. Hopkins' observations on the debranching mechanism and nature of the enzymes specifically affecting amylopectin, because staling associated with starch changes and the reduction of crumb firming by softening agents are inter-related? Were these observations, for example, in any way related to the recently-described effects of the sugar stearates in the crumb softening of bread? How does what we have learned fit in with the supplementation of yeast enzyme action by

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the fungal amylases widely used now in panary fermentation in America and recently described by Dr. Amos in the *J. Sci. Fd Agric.*, 1955, **6**, 489?

To Prof. Conway we are indebted; for many of us considering the British Bread Order, now defunct, have wondered how potassium bromate could safely be regarded as a 'yeast food', when we thought the sole action was the bromate toughening action on the gluten of flour. Now we know that the potassium ion is of great importance to the yeast cell, and that our fears of excusing an illegality were groundless! Nevertheless, this paper and the succeeding paper of Mr. Gilliland emphasized the many factors affecting cell structure and cell proliferation. We might ask, can hybridization in baker's yeast production lead occasionally to mutations where the sugar raffinose is produced? Is that related to the occasional inexplicable crumb stickiness that cannot be associated with high-maltose flours, but which causes the baker added difficulties in the slicing of his bread. The cross-linkage here is with the Plant Breeding Laboratory in Cambridge where I learned that, in the crystallization of beet sugar, traces of raffinose lead to a sticky and most difficultly crystallizable sugar. Do we as cereal scientists tend to look at flour too much, and yeast too little? Should we tackle the problem of panary fermentation more from the point of view of yeast being the main substance, despite its being present at no more than 2% on the flour weight, and regard dough as the culture medium? This is what Dr. McKenzie is doing in his work on flour sugars, but whatever method of approach is used we must remember that yeast *and* flour are both materials of great complexity and no single set of experiments can give a complete picture. This is a pitfall too often ignored by the pure chemist working in this field because by training he regards his reagents always as pure substances and overlooks any possible variation in composition which is the prerogative of naturally-occurring substances.

A symposium should make us pose new questions. Are we, for example, right in the way we examine the quality of baker's yeast at the moment? Are we too wedded to the idea of taking a control flour with a Blish & Sandstedt maltose figure of, let us say, 3 and then comparing the activities of various yeasts by studying the number of ml. of gas produced in the 1st, 2nd, 3rd, 4th, 5th and 6th hours of fermentation. Do we, when we carry out baking tests and look at, say, the rise of a dough in 2 hours, the rise in proof, the height of the loaf and oven spring, examine all the facts we should in assessing yeast quality? It is, of course, easy to convince ourselves that the quality of bakers' yeast in England has improved over the last two

years. We can point to the fact that to produce a standard volume of gas, whereas in 1954 with certain samples of yeast it took 4 lb. of yeast to give equivalent gas in the first hour, $3\frac{1}{2}$ lb. in the 4th hour and $3\frac{1}{2}$ lb. to give equal total gas in 5 hours that the corresponding figures are now $2\frac{3}{4}$ lb., $2\frac{1}{2}$ lb. and $2\frac{1}{2}$ lb. Baking tests involving the measurements just described give the same picture, but have we in fact done enough subjective tests, have we looked at the softness of the crumb, the toughness of the crumb and other factors, such as flavour, which is affected by fermentation? Perhaps such investigations, because of the variability of flour itself, would not give adequate clues, because the statistical interpretation of baking test results are fraught with difficulty. Yet we have seen flavour assessments and some suggestions on the causative chemicals synthesized studied during cider making and learned how the nature of the yeasts and the growth conditions may influence the final result in both beer and cider.

We have heard, too, from Mr. Fowell something of the complexities involved in studying the hybridization of baker's yeasts and from Dr. McKenzie details of the nature of the sugars and changes in their concentration during the fermentation of a strong Scottish flour. Thus, on the one hand there are pointers to studies on improving yeast quality by hybridization, on the other hand one must not forget that the nature and quantity of available sugars will almost certainly vary from grist to grist and that Dr. McKenzie's results are just one example and that Mr. Fowell's hybridization experiments may vary as the composition of his flour nutrient medium varies.

Nevertheless, all these studies point to one thing – are we satisfied with the quality of baker's yeast? I think the nature of the papers read and the discussions give the answer and my reply to this as a scientist is that we are not. What then must we do? My view is that we must look at the whole scope of panary fermentation and the nature of the finished bread more closely and that traditional gassing tests and too conservative baking tests can blind us to what is ultimately required. Thus, although yeast quality has improved in terms of the conventional tests, there are new ideas regarding flavour and bread texture that are closely related to many of the studies reported at this symposium, at first sight unrelated to the bakers' problem, but which I hope in the way I have focused attention on them, have pointed to some topics for still more intensive research. Thus, we might ask the question, can baker's yeast be modified to take into account and contribute more to producing some of the desirable properties in bread to which I have drawn attention?

Can, for instance, other pure microbiological substances be compounded with it, so that during fermentation a number of desirable reactions contributing to bread quality go on side by side?

This brings me to my final point. Bakers and millers everywhere are concerned with the fall in bread consumption. It is no use sheltering behind such fantasies that it is 'boiled bread' that is partly responsible because we at the British Baking Industries Research Association have shown that the temperature conditions in a loaf are no different in the bakers' oven from those in the housewife's cooker. To some extent the popular misconception is a reflection on bread flavour, and that is a property which to a large extent is determined by adequate fermentation. It is here, therefore, that the yeast manufacturers and yeast scientists can play an important part because yeast is vitally important in flavour development. A super-fast yeast would be little good without flavour production, but how much do we know of the part played by specific sugars and the substances synthesized in the production of bread on flavour? We know all too little, but we have now the chemical tools as many of the chromatographic studies referred to in this Symposium indicate, to mention only one line of attack, but one I know that is helping Dr. Cook tremendously in his work and which can help the baker no less. The flavour of the crust may be more related to conditions of baking than to yeast, but the subtle flavour of the crumb is a direct result of adequate panary fermentation. Thus, the yeast manufacturer has a direct responsibility in this matter of bread flavour and we hope he will play his part in maintaining bread sales by research into these matters, which are just as important as gas production and oven spring.

The yeast manufacturer in deriving his yeasts must also be aware of world trends towards either continuous bread-making processes or greater bulk fermentation in terms of the so-called stable ferments being examined particularly in the U.S.A. and which latter have flavour development as a prominent feature in their development programme. It may well be that the trend is towards super-fast yeasts, but the earlier proviso I made must be borne in mind. In addition, just as in the flours which we require for baking purposes generally, where we are moving quickly in the direction of flours tailor-made for specific purposes, it may be that yeasts for specific purposes may also be required – for example, different types for continuous doughmaking from those used by the smaller baker who uses more traditional methods. But in each case it is my belief that there will be a trend towards shorter fermentation times. Applied scientists must always be aware of industrial trends, which are

towards shorter hours of working and plant compression because rateable values and therefore space are becoming more and more expensive. If fall in bread consumption is to be arrested, it must remain a highly competitive food in cost. To achieve this many factors must be borne in mind, but I think there emerges more clearly than ever the part yeast must play, and in that part the over-riding necessity is to help the baker to bake economically *bread with flavour*.

In conclusion, it may be remembered that flour fortification with vitamin B_1 may be doing more than help nutritional standards, because from what we have heard it aids secondary yeast production which may be commercially related to flavour development. We have not, however, noticed any loss of vitamin B_1 during panary fermentation in some recent studies, so we might have accidentally shown a catalytic activity as distinct from a metabolically destructive role, which serves to show we have plenty of real work to do understanding some of these things.

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